Telomere length of kakapo and other New Zealand birds

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assessment of methods and applications

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Abstract

The age structure of populations is an important and often unresolved factor in ecology and wildlife management. Parameters like onset of reproduction and senescence, reproductive success and survival rate are tightly correlated with age. Unfortunately, age information of wild animals is not easy to obtain, especially for birds, where few anatomical markers of age exist. Longitudinal age data from birds banded as chicks are rare, particularly in long lived species. Age estimation in such species would be extremely useful as their long life span typically indicates slow population growth and potentially the need for protection and conservation.

Telomere length change has been suggested as a universal marker for ageing vertebrates and potentially other animals. This method, termed molecular ageing, is based on a shortening of telomeres with each cell division. In birds, the telomere length of erythrocytes has been reported to decline with age, as the founder cells (haematopoietic stem cells) divide to renew circulating red blood cells. I measured telomere length in kakapo, the world largest parrot and four other bird species (Buller's albatross, kea, New Zealand robin and saddleback) using telomere restriction fragment analysis (TRF) to assess the potential for molecular ageing in these species.

After providing an overview of methods to measure telomere length, I describe how one of them (TRF) measures telomere length by quantifying the size distribution of terminal restriction fragments using southern blot of in-gel hybridization (Chapter 2). Although TRF is currently the 'gold standard' to measure telomere length, it suffers from various technical problems that can compromise precision and accuracy of telomere length estimation. In addition, there are many variations of the protocol, complicating comparisons between publications. I focused on TRF analysis using a non-radioactive probe, because it does not require special precautions associated with handling and disposing of radioactive material and therefore is more suitable for ecology laboratories that typically do not have a strong molecular biology infrastructure. However, most of my findings can be applied to both, radioactive and non-radioactive TRF variants. I tested how sample storage, choice of restriction enzyme, gel

electrophoresis and choice of hybridization buffer can influence the results. Finally, I show how image analysis (e.g. background correction, gel calibration, formula to calculate telomere length and the analysis window) can not only change the magnitude of estimated telomere length, but also their correlation to each other. Based on these findings, I present and discuss an extensive list of methodological difficulties associated with TRF and present a protocol to obtain reliable and reproducible results.

Using this optimized protocol, I then measured telomere length of 68 kakapo (Chapter 3). Almost half of the current kakapo population consists of birds that were captured as adults, hence only their minimum age is known (i.e. time from when they were found +5 years to reach adulthood). Although molecular ageing might not be able to predict chronological age accurately, as calibrated with minimum age of some birds, it should be able to compare relative age between birds. Recently, the oldest kakapo (Richard Henry) was found to show signs of reproductive senescence. The age (or telomere length) difference to Richard Henry could have been used to approximate the remaining reproductive time span for other birds. Unfortunately, there was no change of telomere length with age in cross sectional and longitudinal samples.

Analysis of fitness data available for kakapo yielded correlations between telomere length and fledging success, but they were weak and disappeared when the most influential bird was excluded from analysis. The heavy management and small numbers of kakapo make conclusions about fitness and telomere length difficult and highly speculative. However, telomere length of mothers and their chicks were significantly correlated, a phenomena not previously observed in any bird.

To test if the lack of telomere loss with age is specific to kakapo, I measured telomere length of one of its closest relatives: the kea (Chapter 4). Like kakapo, telomere length did not show any correlation with age. I then further assessed the usefulness of molecular ageing in birds using only chicks and very old birds to estimate the maximum TL range in an additional long lived (Buller's albatross) and two shorter lived species (NZ robin and saddleback). In these

species, telomere length was on average higher in chicks than in adults. However, age matched individuals showed high variations in telomere length, such that age dependent and independent telomere length could not be distinguished. These data and published results from other bird species, coupled with the limitations of methodology I have identified (Chapter 2), indicate that molecular ageing does not work in most (if not all) birds in its current suggested form.

Another way to measure telomere length is telomere Q-PCR, a real-time PCR based method. Measurement of the same kakapo samples with TRF and Q-PCR did not result in comparable results (Chapter 4). Through experimentation I found that differences in amplification efficiency between samples lead to unreliable estimation of telomere length using telomere Q-PCR. These differences were caused by inhibitors present in the samples.

The problem of differential amplification efficiency in Q-PCR, while known, is largely ignored by the scientific community. Although some methods have been suggested to correct for differing efficiency, most of these introduce more error than they eliminate. I developed and applied an assay based on internal standard oligonucleotides that was able to corrected EDTA induced quantification errors of up to 70% with high precision and accuracy (Chapter 5). The method, however, failed when tested with other inhibitors commonly found in DNA samples extracted from blood (i.e. SDS, heparin, urea and $FeCl_3$). PCR inhibition was highly selective in the probe-polymerase system I used, inhibiting amplification of genomic DNA, but not amplification of internal oligonucleotide or plasmid standards in the same reaction. Internal standards are a key feature of most diagnostic PCR assays to identify false negatives arising from amplification inhibition. The differential response to inhibition I identified greatly compromises the accuracy of these assays. Consequently, I strongly recommend that researchers using PCR assays with internal standards should verify that the target DNA and internal standard actually respond similarly to common inhibitors.

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1. Introduction

1.1 Telomeres

Muller and McClintock were the first to discover the unique features of the chromosome ends in the late 1930's (see Bolzan & Bianchi 2006 and references herein). Muller found that chromosome repair after radiation-induced double strand breaks never involved the end of the chromosomes in *Drosophila*. He hypothesized "a terminal gene with a special function, that of sealing the end of the chromosome" and named it telomere (from the Greek *telo* = end and *mere* = part). Likewise, McClintock observed that broken chromosomes in maize frequently fuse to their sister chromatids, but normal chromosome ends do not. Both studies demonstrated that the telomeres must have a specialized structure to 'cap' the end of chromosomes and protect them from damage response and fusion.

After discovery of the DNA structure and semiconservative replication the concept of the 'end replication problem' emerged (Watson 1972). It was based on the inability of standard DNA polymerases to replicate the very end of the chromosome (Von Zglinicki 2002; Bailey & Murnane 2006) and the consequence predicted shortening of telomeres in each cycle of DNA replication. Today we know that the end replication problem is only one of many factors contributing to telomere shortening (Bolzan & Bianchi 2006). Other factors counteract shortening by adding pieces to the end of telomeres (Krupp et al 2000; Henson et al 2002).

The first step towards unravelling the molecular structure of telomeres was taken by Blackburn & Gall (1978) who discovered that the telomeres of *Tetrahymena thermophila* were comprised of the tandemly repeated motif TTGGGG. Since then, a repeated G-rich penta- or hexanucleotide motif has been found in most protozoan, fungi, insects, higher plants and animals so far studied (reviewed in Louis & Vershinin 2005; Bolzan & Bianchi 2006). Another part of the puzzle came again from Blackburn working with Greider, (Greider & Blackburn 1985), when they discovered telomerase, an enzyme that can elongate telomeres and hence can solve the end

replication problem. Telomerase carries its own RNA that is used as a template to add new telomeric repeats to the end of the chromosome (Greider & Blackburn 1989). In mammals, telomerase is only active in the germ line, proliferative tissues and cancer cells (Shay & Bacchetti 1997). When Harley et al (1990) finally discovered that telomeres do shorten over time in human cell lines, telomere research quickly expanded into cancer and ageing research (Bolzan & Bianchi 2006, see below).

All vertebrates (Moyzis et al 1988; Meyne et al 1989) and several invertebrates (Lejnine et al 1995; Plohl et al 2002) share the telomere motif TTAGGG. The length of the telomere array varies between species (reviewed in Louis & Vershinin 2005), individuals (Rufer et al 1999; Baerlocher et al 2007), gender (Kimura et al 2007) and even chromosomes (Lansdorp et al 1996; Zijlmans et al 1997; Londono-Vallejo et al 2001; Baird et al 2003). Another important part of the telomere is the G-rich single stranded overhang at the 3' end (reviewed in Bertuch & Lundblad 2006). Three possible mechanisms have been proposed for the generation of the overhang (Louis & Vershinin 2005): I) incomplete synthesis of the telomeric C-strand by lagging strand DNA replication, II) synthesis of G-overhangs by telomerase and III) nucleolytic degradation of the C-strand. The overhang is believed to fold back into the double stranded telomere region to form a t-loop that is an important substrate for protein binding (Figure 1.1; reviewed in Bailey & Murnane 2006; Bolzan & Bianchi 2006). DNA binding proteins, like TRF1 and TRF2, bind directly to the telomereic repeats and recruit additional proteins (Figure 1.1). Six main proteins have been proposed to form a complex called shelterin that caps the end of the chromosomes (de Lange 2005).

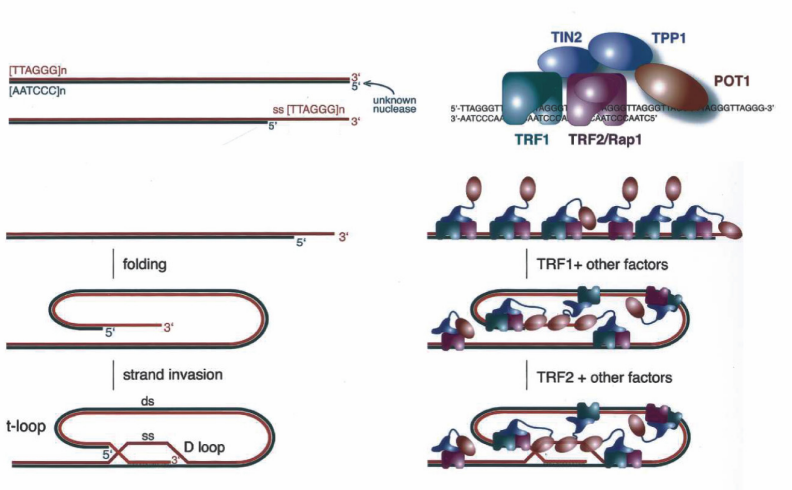

Figure 1.1: Structure of telomeres. Picture from and model proposed by de Lange (2005). After replication an unknown nuclease creates a long G-strand overhang that can invade double stranded DNA and form a t-loop. Several proteins assist in the invasion, stabilization of loop confirmation and regulation of accessibility for telomerase. For more information see de Lange (2005).

The main function of telomeres on the cellular level is to protect the chromosome end from degradation, recombination or fusion with other chromosomes that could result from activation of DNA damage response machinery (de Lange 2002). In addition, telomeres are important for proper positioning of chromosomes during replication (Kirk et al 1997) and they can reversibly repress expression of subtelomeric genes (Baur et al 2001; Mefford & Trask 2002; Louis & Vershinin 2005). Recently it has been found that telomeres are actually transcribed and the telomeric repeat containing RNA (TERRA) remains partly associated with the telomeres (Azzalin et al 2007). The authors proposed the involvement of TERRA in telomere replication homeostasis, but further investigation was suggested.

On an organism level telomeres have been found to regulate replicative capacity mediated by their length. Due to the intrinsic ‘end replication problem’ and a variety of extrinsic factors (see below) telomeres shorten every time they are replicated (Cimino-Reale et al 2003). If the telomere length decreases below a certain threshold cells usually enter senescence (Harley et al 1990). Passing this check point usually results in apoptosis or genomic instability and massive, often lethal, chromosome rearrangements (reviewed in Bolzan & Bianchi 2006). Telomere length therefore limits the replicative capacity of cells (Allsopp et al 1992; Martens et al 2000).

These findings led to the theory of ageing and cancer (reviewed in Klapper et al 1998; Bell & Sharpless 2007) and the idea of the disposable soma (Wright & Shay 2005) :

The limitation of potential cell division to a finite number is thought to be a mechanism to protect the whole body from cancer, a disease caused by uncontrolled cell divisions. The development of cancer requires several mutations that are acquired mainly during DNA replication (Wright & Shay 2005). Critically shortened telomeres induce senescence or apoptosis in cells that underwent enough replications to potentially develop cancer. While this provides some protection against cancer, these cells are excluded from the pool of cells needed to regenerate tissues. This lack of regeneration is the beginning of what is termed ageing. Wright & Shay (2005) calculated that every human somatic cell has just enough replicative capacity to maintain optimal body function to the age of approximately 40 years. Under this hypothesis, the price for protection against cancer is ageing. Only the germ line is immune to this mechanism as it exhibits telomerase activity to ensure proper telomere length for the next generation. The body itself is only a vessel for the germ line (disposable soma).

The above theories were supported by the early finding that telomerase activity is necessary for a cell to become immortal in cell culture (Counter et al 1992). We know now that telomerase activity can be found in approximately 80% of all tumours and immortal cell lines (reviewed in Shay & Bacchetti 1997; Bailey & Murnane 2006; Bolzan & Bianchi 2006). The other 20% maintain their telomere length by alternative lengthening of telomeres (ALT) (Hande et al 1999; Henson et al 2002; Teixeira et al 2004; Marciniak et al 2005), mainly by recombination (reviewed in Bhattacharyya & Lustig 2006). Telomerase activity is probably regulated by the accessibility of telomeres. Teixeira et al (2004) found that telomere length maintenance by telomerase in yeast is an equilibrium between the absence and presence of telomerase activity on each telomere. The amount of added nucleotides was independent of the length of the telomere (except in ultra short telomeres), but the event of elongation itself had a higher frequency for shorter telomeres. The authors suggested that telomere length regulates the

affinity with which telomerase binds to telomeres, with shorter telomeres promoting easier access. In fact, human cell lines show a preferential elongation of short telomeres (Ouellette et al 2000; Londono-Vallejo et al 2001). The action of telomerase is also affected by external factors like oxidative stress (Colitz et al 2004) or smoking (Getliffe et al 2005) and telomerase may also have an anti-ageing effect through a pathway independent of telomere length (Geserick & Blasco 2006).

Although telomerase is active in the human germ line, stem cells and lymphocytes, activity sufficient to maintain telomere length is found in the germ line only (Goronzy et al 2006). Consequently, all other tissues show telomere shortening over time/cell divisions. The rate of shortening is influenced by several factors (reviewed in Lansdorp 2005), including oxidative stress (Von Zglinicki 2002), activity of exonucleases to generate the 3' overhang (Makarov et al 1997) and even psychological stress in humans (Epel et al 2004).

Telomere length is also a partly heritable trait (reviewed in Baird 2006; Baird 2008) with more than a hundred genes found in yeast influencing telomere length (Askree et al 2004). Interestingly, inbred populations of mice and rabbits have longer telomeres than outbreed populations, with inbreed mice having several times more telomeric DNA (Kipling & Cooke 1990; Prowse & Greider 1995; Manning et al 2002; Forsyth et al 2005). There is also a significant difference between telomere length of different inbreed mice strains (all possessing extraordinarily long telomeres), suggesting a substantial influence of genetic deposition on telomere length at least on a population level (Manning et al 2002). Within populations various degrees of heritability have been estimated (Slagboom et al 1994; Jeanclos et al 2000; Andrew et al 2006; reviewed in Baird 2006) and paternal (Nordfjall et al 2005) and maternal (Nawrot et al 2004) linkage has been reported. Most genetic determinants of telomere length found so far were associated with telomerase (Baird 2008), but many pathways of telomere length change and their molecular basis are still waiting to be unravelled.

The clinical applications of telomere length have initially been focused on diagnostics of cancer (Meeker et al 2002; Bisoffi et al 2006) and correlation with human chronic diseases and premature ageing syndromes (Geserick & Blasco 2006). Later studies focused also on the effect of telomere length corrected for age (age-independent telomere length) on survival rate and the onset of age dependent diseases. Age independent telomere length seems to be a marker for mortality in elderly humans in some cases (Cawthon et al 2003; Martin-Ruiz et al 2006), but not in others (Martin-Ruiz et al 2005; Bischoff et al 2006). Furthermore, telomere length of leucocytes is correlated with a variety of age dependent diseases, like coronary heart disease, hypertension or dementia (reviewed in Aviv 2006). More recently, another branch of telomere research has emerged linking ageing research with evolutionary ecology. It started as a ageing tool (Haussmann & Vleck 2002) and is now being trialled, with varying degrees of success, as a marker for fitness of individuals in wild populations (Haussmann et al 2005; Pauliny et al 2006).

1.2 Molecular ageing

Although telomere length (TL) has been previously linked to age in some mammals (human (Frenck, Jr. et al 1998), domestic cat (Brummendorf et al 2002)), the concept of molecular ageing was introduced into ecology by Haussmann & Vleck (2002). They showed that age correlates with telomere length of blood cells in captive Zebra Finches (*Taeniopygia guttata*) and can explain approximately 50% of individual TL. Based on this observation, they suggested that blood TL could be used to age individuals of wild bird populations, if enough birds of known age are available to construct a standard curve. Such a tool would be very useful to study ecological and evolutionary traits in natural populations where longitudinal data are typically absent. Knowledge of age structure of a population may be vital to interpretations of life history parameters (Haussmann & Vleck 2002 and references therein; Monaghan & Haussmann 2006).

For studies on birds, blood is a very good tissue to measure TL, because it is taken routinely in the field for other reasons, such as parasite screening, population genetic analysis

etc. and sampling is non destructive and only moderately invasive. As bird erythrocytes are nucleated, one drop of blood (approximately 10µl) contains enough DNA for repeated measurements of TL. Also, red blood cells have a high turn over rate (approximately 30-40 days in chicken, pigeon and duck (Rodnan et al 1957)), which means they originate from stem cells that are highly replicative and hence presumably show a high telomere rate of change (TROC) (Haussmann & Vleck 2002).

The general concept of molecular ageing was confirmed by the same authors in four additional bird species with coefficients of determination (R^2) between 34% and 66% (published twice : Vleck et al 2003; Haussmann et al 2003b). In addition, a tight correlation between TROC and maximal lifespan of species was found, with one long lived species, the Leach's Storm-petrels (*Oceanodroma leucorhoa*), even showing an increase of telomere length with time. A subset of the same data has been used to show that TL can be used to predict broad age classes (<6 yr. and >12 yr.) in common terns (*Sterna hirundo*) (Haussmann et al 2003a).

Other authors have tested Haussmann & Vleck's method with varying success. Hall et al (2004) found a significant change of TL only between chicks and adult birds of the European shag (*Phalacrocorax aristotelis*) and the wandering albatross (*Diomedea exulans*), but not between adult birds of different age. Likewise, Pauliny et al (2006) reported the highest R^2 for sand martin (*Riparia riparia*) and dunlin (*Calidris alpine*) when using an power fit rather than a linear curve, indicating the high difference between chicks and adults. Both studies also showed using longitudinal samples, that TROC is not constant throughout lifetime, but changes with age. The phenomena of varying TROC between early life and adulthood has also been found in humans (Frenck, Jr. et al 1998; Rufer et al 1999; Zeichner et al 1999), baboons (Baerlocher et al 2007) and domestic cat (Brummendorf et al 2002). Taken together, these studies indicate that a correlation between TROC and maximum life span must be interpreted carefully (Hall et al 2004; Monaghan & Haussmann 2006).

The first (and only) test of molecular ageing was conducted in frigatebirds (*Fregata minor*) (Juola et al 2006), where the authors used 36 birds of known age to construct a calibration curve for ageing unknown birds. Although TL declined faster in younger birds, there was still substantial loss of telomeric repeats with age in adults. The authors were able to obtain a coefficient of determination of 0.82 for their regression after square root transforming age. This curve has then been used to age 102 female breeders of unknown age. Due to the high variation of TL between similar aged birds used to construct the standard curve, the 95% confidence intervals were so large, that no reliable assignment of age could be achieved. Some birds were assigned to an age class of 72 yr. and 205 yr. (95% confidence interval) although the maximum reported lifespan of this species is 44 years. While this species might not have been an optimal choice for molecular ageing, because of high TL variation between individuals and low TROC in adults, the R^2 of 0.82 is better than all coefficients of determination reported in previous studies.

The main factor compromising a reliable estimation of age is probably the high variation between individuals of the same age. Scott et al (2006) proposed to use TL to estimate the relative age of American alligators (*Alligator mississippiensis*) of the same body length. Following this suggestion, TL might be useful if measured in combination with other age linked markers like length, weight or plumage. Comparison of TL within age classes defined by these additional markers might reveal the age of individuals relative to each other. However, differences in TL might not necessarily reflect chronological age, but biological age instead, because, as discussed above, various factors influence TL and TROC. In birds telomere length as a chick, birth date within the season and body mass as chick have been found to strongly influence telomere loss (Hall et al 2004), the later two probably via oxidative damage. Age independent TL might therefore be a marker for stress experienced in the past, or an indication of an individual's ability to cope with stress. This assumption leads to a possible correlation of TL and fitness. Birds with longer telomeres have been shown to have a higher survival rate even early in life (Haussmann et al 2005) and are believed to have higher reproductive success

(Pauliny et al 2006). It has also been suggested that longer telomeres are under selection in Leach's storm petrel (*Oceanodroma leucorhoa*) (Haussmann & Mauck 2008b). Here, adult birds appear to have longer telomeres than most chicks. The authors believe that TL does declines with age, but birds with shorter telomere length have a lower survival rate. Consequently, birds returning to the colony as adults derive from chicks with very long telomeres and still have longer TL than the average chick. This theory awaits further investigation, but if telomeres are under selection, this is another factor that can distort the correlation of telomere length and age in cross-sectional studies.

There is a substantial amount of inconsistency in the methodology to measure telomere length, both between and within research groups over time. Especially, the proportion of telomere smear measured using telomere restriction fragment (TRF, see Chapter 2) analysis differs between publications (Haussmann et al 2003b; Hall et al 2004; Pauliny et al 2006; Haussmann & Mauck 2008a). In some cases it is unclear which part of the smear has been used as authors contradict themselves in different publications (e.g. Haussmann & Vleck 2002; Haussmann & Mauck 2008a). Haussmann & Mauck (2008a) made an attempt to standardize sample storage and the method to measure telomere length. This approach did not work in the sample sets used in the present study and is discussed further in section 2.2.5.5.

This thesis investigated whether TL measurement is a valid tool for ecological studies. Therefore, different methods to measure telomere length were analysed and their feasibility for ecological orientated (and equipped) laboratories was assessed (Chapter 2). The main species of investigation was the kakapo (*Strigops habroptilus*), a highly endangered parrot endemic to New Zealand (Chapter 3). In addition, several other NZ birds have been tested for telomere length change (Table 1.1, Chapter 4). An additional data set from a fish species (European sea bass (*Dicentrarchus labrax*)) collected prior to this PhD has been included, because it was essential for error assessment of different analysis methods and has not been published before. Results of the sea bass study are shown in Appendix III. The work using quantitative real-time PCR lead

me from applied research to the field of basic research about the fundamental features of PCR (Chapter 5 and 6).

Table 1.1: Species used in this study.

Species	**Scientific name**	**sample size**	**sample range [years]**
Buller's albatross[a]	*Thalassarche bulleri*	10	0-21[c]
European sea bass[b]	*Dicentrarchus labrax*	24	1-8
kakapo	*Strigops habroptilus*	68	1-35[c]
kea	*Nestor notabilis*	20	0.5-27
NZ robin[a]	*Petroica australis*	10	0-2
saddleback[a]	*Philesturnus carunculatus*	10	0-5.5
zebra finch	*Taeniopygia guttata*	20	0-2.5

[a] no/few intermediate aged individuals. [b] Samples and data collected at IFREMER-Station Palavas Les Flots, France.
[c] minimum age in adults, estimated from birds banded as adults.

This thesis was a progressive work and is presented as such.

Chapter 1 gives a brief introduction about telomeres and the concept of molecular ageing: estimating the age of individuals based on their telomere length.

Chapter 2 gives an overview of the methods currently used to measure telomere length and explains the choice of methods employed in this study. Two methods were chosen to be investigated further: telomere restriction fragment (TRF) analysis and quantitative PCR (q-PCR). TRF is further explained and various pitfalls and ways to avoid them are shown. Q-PCR did not result in reliable estimates of telomere length but lead to an exploration the fundamental nature of PCR and is further discussed in Chapters 5 and 6.

Chapter 3 presents telomere length of kakapo measured by TRF and discusses the applications of telomere length for kakapo recovery.

Chapter 4 presents additional telomere length data for four New Zealand bird species and discusses the utility of telomere length as a marker for age and fitness in birds in general using data from the present study and previously published data.

Chapter 5 presents telomere data of kakapo obtained by Q-PCR and shows how differences in amplification efficiencies between samples can compromises reliability of results obtained with this method.

Chapter 6 gives an overview on PCR inhibition, the main cause if variations in amplification efficiencies and presents an experimental approach to correct for differential efficiencies.

Chapter 7 summarizes the findings of the present study and discusses their application to conservation, wildlife management and diagnostic PCR.

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2. Methods to measure telomere length

This Chapter gives a brief introduction to different methods to measure telomere length. The main focus lies on their measurement error and their feasibility for ecological studies. The most commonly used method, telomere restriction fragment (TRF) analysis, is further described in detail. A variety of measurement error sources are explained in experimental examples and a protocol is developed to ensure maximum reproducibility of telomere length estimates.

2.1 Overview

Telomeres do not have a defined border at the proximal end (Nakagawa et al 2004). Fig. 2.1 shows the different parts involved in the architecture of telomeres starting from the subtelomeric region over imperfect telomeric repeats to the true telomeric repeats and finally the single stranded G-rich 3' overhang. The actual size of the regions differs between telomeres, chromosomes and species. Several methods have been developed that measure different parts of the array as indicated in Fig. 2.1.

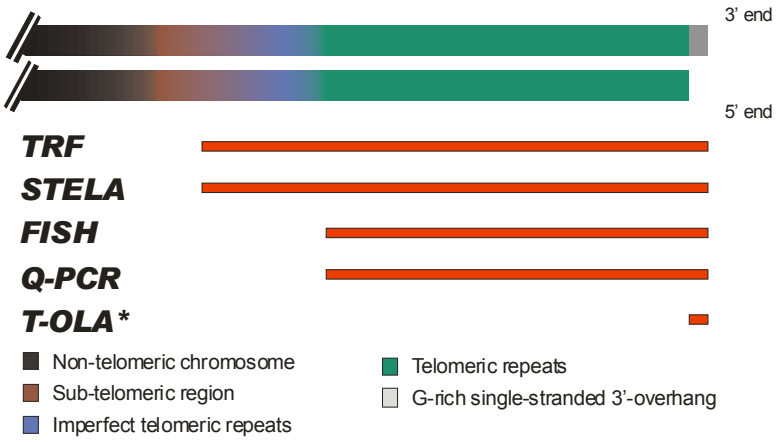

Figure 2.1: Structure of telomeres and sections measured by different techniques (red bars). TRF = Telomere Restriction Fragment; STELA = Single Telomere Length Analysis; FISH = Fluorescent In Situ Hybridization; Q-PCR = Quantitative Polymerase Chain Reaction; T-OLA* = Telomere Oligonucleotide Ligation Assay, representative for several methods measuring G-rich overhang (see text). The size of the regions differ between telomeres, chromosomes and species. Adapted and modified from Nakagawa et al (2004).

The first report of telomere length measurement goes back to 1990 when Harley and colleagues investigated the change of telomere length (TL) in cultured human fibroblasts with ongoing cell replications (Harley et al 1990). They cut genomic DNA with the restriction enzymes *Msp*I and *Rsa*I to generate terminal restriction fragments and used a conventional Southern blot procedure with a radioactive probe to detect the fragments containing telomeric sequences. This method was called telomere restriction fragment (TRF) analysis and is still widely used (see Appendix Table II.I). Many variations of this method have been described, but they all follow the same principle. A detailed description of TRF and its variations is given in Section 2.2. Several alternative methods to measure TL have also been developed (reviewed in Saldanha et al 2003; Nakagawa et al 2004; Baird 2005, Fig. 2.1 and Table 2.1) and are discussed below.

2.1.1 Fluorescent in situ hybridization (FISH)

Like TRF, fluorescence in-situ hybridization (FISH) is a hybridization based method. A fluorescent labelled probe is hybridized to DNA in fixed cells and detected using a fluorescence microscope. The first attempts to measure TL by FISH utilized fluorescent labelled DNA probes (Henderson et al 1996). Although no precise signal quantification could be obtained, the authors could correlate measures like signal detection frequency, signal intensity and spot size with the rate of replicative ageing in several cell lines. A new method, called quantitative FISH (Q-FISH), was developed with the introduction of peptide nucleic acid (PNA) hybridization probes. PNA oligonucleotides hybridize to complementary nucleic acid with higher affinity than DNA/DNA or DNA/RNA hybrids (Egholm et al 1993) and provide the necessary precision to quantify the telomere signal of individual chromosomes in metaphase spreads. Since its first introduction (Lansdorp et al 1996), telomere Q-FISH has been used widely and has been shown to correlate with TL obtained by TRF (Lansdorp et al 1996; Meeker et al 2002; Ferlicot et al 2003). The main advantage of Q-FISH is that this technique is able to measure the TL of individual

chromosomes. As all studies to date showed substantial heterogeneity in TL between different chromosomes and chromosome arms, Q-FISH is the method of choice to study the influence of individual telomeres on cell senescence and apoptosis. The disadvantages include that a full TL analysis can only be done in culturable or rapidly dividing cells, as individual telomeres can only be resolved in metaphase spreads. A reliable error assessment is difficult because every cell can only be hybridized and measured once making replicates impossible. A 40-50% signal variability has been reported between metaphase spreads on the same slide (Londono-Vallejo et al 2001), but was reduced to approximately 10% by dividing the signal of each chromosome by the total signal from all telomeres of the measured cell. Alternatively, the signal strength of sister chromatids has been used to validate Q-FISH, as they are expected to have the same TL. A good correlation has been found between TL of sister chromatids (Lansdorp et al 1996; Nagele et al 2001), but also up to 50% difference in some studies, suspected to be due to methodological errors (Zijlmans et al 1997). Therefore more than one metaphase spread should be analysed to obtain a reliable mean TL for a given cell type. Similarly, Londono-Vallejo et al (2001) reported a 15-20% fluctuation in mean signal intensity per cell within the same slide. When testing different cell lines, this study also found a good correlation (R^2=0.90) between TRF results and the mean fluorescence intensity derived from 15-25 cells per cell line. However, the standard deviation of the fluorescent intensity for some cell lines is higher than the complete range of measured telomere length (approximately 8 kb). It is not clear if the observed deviation is due to heterogeneity between individual cells of the same cell line or measurement error. For example, the definition of telomere boundaries has been found to influence the reproducibility of Q-FISH measurements (Nagele et al 2001). Either way, the strength of FISH lies in the ability of resolve individual chromosomes and not to measure the mean TL of a tissues.

Several modification have been developed to overcome the restrictions of the traditional Q-FISH, namely the need to cultivate cells to obtain metaphase spreads. Q-FISH has been used to measure TL in tissue sections, but only a subset of telomeres could be measured. Ferlicot et al

(2003) reported the detection of 12-22 out of 92 telomeres per cell in human liver, kidney and thyroid sections. In other studies single telomeres could not be distinguished and the total telomere signal per cell was normalized to centromere signal, DNA counterstaining or telomere signal of other cell types in the same section (O'Sullivan et al 2002). In any case, a high number of cells has to be examined to obtain a reproducible mean telomere length for a given tissue (Ferlicot et al 2003). Q-FISH on tissue sections has also been combined with antibody staining to identify TL of specific cell types in tissue samples (Meeker et al 2002). Here, the telomere signal was normalized to 4'-6-diamidino-2-phenyllindole (DAPI) staining. Variations of 7% to 12% have been reported for tissue sections using serial sections of the same tissue block (Ferlicot et al 2003).

2.1.2 Flow-FISH

The combination of Q-FISH with flow cytometry led to a method called flow-FISH (Rufer et al 1998). This method can identify different cell types and measure their TL simultaneously. It has been used mainly to examine telomere dynamics in different cells of the immune system (Baird 2005). The high throughput potential was demonstrated in a study on peripheral blood mononuclear cells (PBMC) of more than 500 human samples (Rufer et al 1999). In this study a variation coefficient of 15% was reported for one sample analysed in each experimental run. Another disadvantage is that the distribution of TL can not be determined by flow-FISH. A study on inbreeding in mice showed that the offspring of a cross between inbreed mice with long telomeres and outbreed mice with short telomere possess an intermediate TL, but it could not be determined if the different TL of the parents have been conserved in both homologous sets of chromosomes, or if all telomeres have been readjusted to an intermediate TL (Manning et al 2002). A longitudinal study following TL change in cat (*Felis domesticus*) over the period of one year also revealed "considerable variation in subsequent samples from the same animals … indicative of method-related variability" (Brummendorf et al 2002).

2.1.3 T-OLA and other methods for measuring G-rich overhang

Several methods have been designed to measure the length of the 3' single-stranded G-rich overhang. Early studies used hybridisation signal strength (Hemann & Greider 1999; Keys et al 2004), electron microscope pictures of purified telomeres (Wright et al 1997; Huffman et al 2000) or primer-extention/nick-translation reaction (PENT) (Makarov et al 1997) to assess the overhang length. A simpler and more precise method called telomere oligonucleotide ligation assay (T-OLA) has been introduced by Cimino-Reale et al (2001). It is based on small oligonucleotides that can hybridize to the single stranded overhang. If two or more of them hybridize next to each other, they are joined by the enzyme ligase and the length of the resulting fragment is proportional to the length of the overhang. As different telomeres have different overhang lengths, the result is a ladder of multiples of the oligonucleotide length that can be analysed quantitatively.

Other alternatives have been developed more recently. In the overhang protection assay (Chai et al 2005) the proteins gp32 and UP1 are bound to the single stranded overhang and protect them during a DNase I treatment that digests all non-protected DNA. After a second digestion with Proteinase K only the overhangs are left and can be analysed by electrophoreses. The G-tail telomere hybridization protection assay (Gt-telomere HPA) uses a probe that is labelled with chemiluminescent acridinium ester (Tahara et al 2005). After hybridization to the overhang, excess and misannealed probe is deactivated by hydrolysis and only bound probe emits light. The signal can then be normalized to the total DNA by a reference sequence (eg *Alu*). Another method uses a double strand specific nuclease (DSN) to purify the single stranded part of the telomere and the resulting fragments can be analysed by southern blot (Zhao et al 2008).

The results obtained are not consistent between the different methods (Chai et al 2005), but a constant overhang length during the replicative stage of cells followed by a abrupt drop at senescence has been found repeatedly in cell culture (Stewart et al 2003; Hashimoto et al 2005;

Tahara et al 2005). Although a correlation between rate of telomere shortening and overhang size has been proposed initially by electron microscope analysis (Huffman et al 2000), newer studies could not confirm this finding (Keys et al 2004). Also, different lengths between leading and lagging daughter strands have been found, suggesting different regulation mechanisms for both ends of the chromosomes (Chai et al 2006). Taken together, these contradictory findings suggest that single stranded G-rich overhangs are not likely to be a appropriate marker for telomere shortening or ageing.

2.1.4 Single telomere length assay (STELA)

Single telomere length assay (STELA) was the first PCR based method to measure TL (Baird et al 2003). It requires a known sequence in the distal part of the subtelomeric region that can be used as a primer binding site. A second binding side can be generated by ligating a linker containing a suitable sequence onto the G-rich overhang. These two binding sites are used for a PCR spanning the whole telomere and a part of the subtelomeric region. STELA has been found to correlate with TRF with coefficients of determination (R^2) of 0.72 (Kimura et al 2007) and 0.82 (Baird et al 2006), but the latter value is not supported by independent calculation from the data presented in the Table of this publication. The advantage of STELA is the high throughput potential and the specificity to one chromosome arm. On the other hand it is very difficult to obtain subtelomeric sequences close enough to the telomeres to span the distance by PCR (Baird, personal communication). STELA has been established for human chromosome XpYp (Baird et al 2003; Baird et al 2004; Baird & Kipling 2004); 17p (Baird et al 2006); 2p, 11q and 12q (Britt-Compton et al 2006) and chromosome V of *Caenorhabditis elegans* (Cheung et al 2004).

2.1.5 Telomere quantitative real-time PCR

Another PCR approach was developed by Cawthon in 2002. Due to the repetitive nature of telomeres they were thought to be not replicatable by PCR, because primers for these repeats would anneal to each other. Cawthon designed a set of primers with several mismatches that inhibit primer dimmer but still shows enough affinity to telomeric sequences to amplify pieces of telomeres. The amplification was shown to be proportional to the amount of telomeric DNA in a sample and can be measured by quantitative real-time PCR (Q-PCR). To control for the start amount of DNA in each reaction the telomere signal was normalized to a single copy gene. Q-PCR measurements of TL have been shown to correlate with values obtained by TRF (R^2=0.677, Cawthon 2002). This technique has an extremely high throughput capacity, but the ease of generating data by real-time PCR can lead to questionable results when scientists do not follow basic rules of real-time PCR (Horn T , in press). A detailed explanation of telomere Q-PCR and the experimental difficulties that can arise using this method is given in Chapter 5.

Several other assays have also been developed that measure the amount of telomere signal obtained by various detection systems and normalize it to DNA concentration (Freulet-Marriere et al 2004), centromeres (Bryant et al 1997) or *Alu* sequences (Aikata et al 2000), but have not been explored in this study.

Traditionally, all methods developed since TRF have been examined for their correlation with TRF. However, a common mistake arising from such comparisons of methods is to confuse significance of correlation with goodness of fit. A significant correlation with an R^2 of 0.36 (Ferlicot et al 2003) does not support the hypothesis that both methods measure the same telomeres length. Furthermore, every new technique is claimed to be more accurate than TRF, but is validated by showing that it generates basically the same TL data as the TRF approach.

2.1.6 Choice of method

All the techniques reviewed above have their advantages and disadvantages. An overview of these is given in Table 2.1. The ratings are somewhat subjective and might differ from the opinion of some colleges. The aim of this study was to investigate if TL can be used for ageing kakapo and other NZ birds from blood samples. If so, it could find wide use among ecologists that are not necessarily interested in molecular biology and other genetic applications. Therefore the main criteria for choice of methods were:

1) no need for specialized (and expensive) equipment
2) high throughput potential
3) no need for prior genetic information

Both FISH methods require specialized microscopes/flow cytometry detectors and experience in handling and cultivating cells. Information obtained in addition to mean telomere length, like TL of individual cells or telomeres, or the ability to distinguish cell types in samples are very useful for medical investigations and research on cancer and ageing, but are not likely to increase the precision when ageing whole animals.

Some applications measuring the length of the G-rich overhang do meet the criteria outlined above, but it is not likely that the G-rich overhang changes length with age. Nevertheless some attempts have been made to simplify the T-OLA technique and enable a high throughput telomere length measurement via fragment analysis on a capillary sequencer. Unfortunately the amount of ligation product obtained in this trail was below the detection level (data not shown). STELA requires a known subtelomeric primer binding side, which appears to be species specific and difficult to obtain (Baird, personal communication).

Table 2.1: Properties of different methods to measure telomere length. TRF = Telomere Restriction Fragment; STELA = Single Telomere Length Analysis; FISH = Fluorescent In Situ Hybridization; Q-PCR = Quantitative Polymerase Chain Reaction; T-OLA* = Telomere Oligonucleotide Ligation Assay, representative for several methods measuring G-rich overhang (see text). † Some techniques for G-rich overhang enable high throughput. Adapted and modified from (Nakagawa (2004) and Baird (2005)).

Method	Hybridization or PCR	Amount of cells required	Range of cell types	Through-put	Specialized equipment	TL individual Cells	TL individual telomeres	TL distribution
TRF	Hyb	+++	+++	++	+	-	-	+
Q-FISH	Hyb	++	+	+	++	+	+	+
flow-FISH	Hyb	++	+	+++	+++	+	-	-
T-Ola*	Hyb	+++	+++	+++†	+	-	-	-
STELA	PCR	+	+++	+	+	-	+	+
Q-PCR	PCR	+	+++	+++	+	-	-	-

The remaining techniques, namely TRF and Q-PCR, have been explored and optimized for reproducibility. The Q-PCR approach resulted in the discovery of significant error sources in the basic approach and interpretation of the Q-PCR method and is discussed in detail in Chapter 5 and 6.

TRF has already been used in several ageing studies on birds (Haussmann & Vleck 2002; Haussmann et al 2003a; Haussmann et al 2003b; Hall et al 2004; Pauliny et al 2006; Juola et al 2006) and reptiles (Scott et al 2006). It has intermediate throughput capacity and can be performed with a minimum of genetic laboratory equipment. TRF can be valuable technique if a number of technical aspects are take into account. The next section reports on how different steps of the TRF protocol can influence the results and how reproducible telomere length values can be obtained.

2.2 Telomere restriction fragment (TRF) analysis

Telomere restriction fragment (TRF) analysis was the first method to be used to measure telomere length (Harley et al 1990). Although TRF actually measures the mean length of the telomere fragment, comprised of true telomeres and a subtelomeric region (Fig 2.1), this length it is usually referred to as mean telomere length (TL). For simplicity, this convention will be followed throughout the present study. The impact of the subtelomeric region on measured TL is discussed in Section 2.2.2.

A variety of modifications of TRF have been developed. The main differences between the variants are the location of hybridization, the hybridization target and the detection method. All methods start with a restriction of genomic DNA with restriction enzymes to obtain telomere restriction fragments, followed by electrophoresis. A radioactive probe can be hybridized to either only the G-rich overhang or the entire telomere. For the entire telomere a denaturing step is necessary to make the DNA accessible for the probe. Hybridizing only to the overhang has the advantage that interstitial telomeric repeats, which sometimes interfere with calculation of TL, are not detected. The hybridization can be done directly in the gel (in-gel hybridization) or after transferring the DNA to a membrane (Southern Blot). After washing away excess probe the signal is detected by x-ray film or phosphorimager. TRF with radioactive probe requires fewer manipulations of the gel or blot, but takes longer due to the detection mode with exposure times of over night up to several days. Depending on the country, handling of radioactive material requires specialized facilities and training. Also, radioactive probes have a limited life span due to radioactive decay. The alternative, Digoxygenin (Dig) or Biotin labelled probes, can be stored for years in the freezer and are safe to use in a normal laboratory environment, but care must be taken not to introduce artefacts during the additional detection steps. To obtain sufficient signal with these probes the DNA has to be denatured after electrophoresis. In-gel hybridization has been reported for the Dig system (Khan et al 1999), but the quality of the images presented suggests a qualitative use of this method rather than a quantitative measurement of the signal as

required for TRF. After Southern blot and hybridization, the probe is detected by chemiluminescence. Usually an antibody coupled to the enzyme alkaline phosphatase is bound to the probe and a chemiluminescent substrate is used to generate the signal detected by x-ray or chemiluminescence film. Washings after hybridization and antibody binding are the most critical steps of this procedure because they determine the amount of background and artefacts due to unspecific binding. Exposure times for chemiluminescence detection are usually between a few seconds and several minutes. This makes this technique much faster than radioactive Southern blot. Also, pictures with different exposure times can be taken immediately. More details on chemiluminescence detection can be found in various manufactures manuals (e.g. 'DIG Application Manual for Filter Hybridization, Roche').

Non-radioactive chemiluminescent Southern blot was chosen for this study because of the advantages for use in ecology and wildlife management. If an assessment of individuals or a small group of animals is necessary, a small amount of Dig-probe can be used immediately in contrast to radioactive probes that have to be ordered freshly and potentially in quantities too great to be efficiently used before their half-life expires. No special equipped laboratory and training is necessary and the detection is faster. However, due to the increased amount of handling during the detection process, a careful optimization is necessary to ensure reproducibility and reliability. A detailed description of the optimized method developed in the present study is given in Appendix I.III.I. Here, some experimental steps will be highlighted and it will be show how they can influence the results and how the resulting measurement errors can be minimized.

2.2.1 Sample storage

One of the most cited disadvantages of TRF is the requirement of high quality DNA (Saldanha et al 2003; Baird 2005). Haussmann et al (2008) suggested that lysis buffer like Queen's lysis buffer (Seutin et al 1991) are not suitable for storage of blood samples used for

TRF because of DNA degradation and refers to Freed et al (2006). The Freed study is a meta-analysis of different publications about detection limit for avian malaria using PCR. The authors concluded that lysis buffer increases the detection limit (lower sensitivity) of diagnostic PCR assays, but no clear connection could be drawn between the use of lysis buffer and degradation of DNA. The effect they found could be due to inhibition of PCR that can arise from multiple factors (reviewed in Wilson 1997), including substances contained in many of the standard lysis buffers, such as SDS and EDTA (see Chapter 6). SDS, which is present in lysis, but absent from non-lysis buffers is a strong PCR inhibitor (Weyant et al 1990; Rossen et al 1992, present study). The higher detection limit with lysis buffer is probably due to chemical interactions during PCR leading to lower amplification efficiency and not due to DNA degradation. To test the influence of sample storage, blood samples of three kakapo were taken and half of it was frozen immediately in liquid nitrogen while the other half was diluted in Queen's lysis buffer. The frozen samples were kept for approximately one week in nitrogen and were subsequently stored another week at -80°C. The lysis buffer samples were kept approximately two weeks at room temperature.

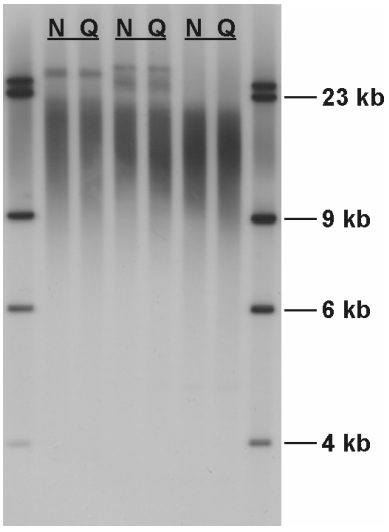

Figure 2.2: Influence of sample storage on telomere length estimate. Blood of three different birds (underline) has been stored for approximately 2 weeks on liquid nitrogen/-80°C freezer (N) or Queens lysis buffer (Q).

All samples were extracted alongside using the same protocol and TL was measure by TRF. Fig. 2.2 shows the TRF smears of all birds for liquid nitrogen/-80°C and lysis buffer storage. All samples were measured twice. The mean TL difference between the two storage methods was 332 bp or 2.12%. This value is well below the intra-gel variation of 616bp or 3.96% (Table 2.5, lga). Short term storage in Queens lysis buffer at room temperature seemed not to influence the measured TL compared to liquid nitrogen/-80°C storage.

2.2.2 Restriction

TRF measures the mean length of restriction fragments that are comprised of the true telomeres and a subtelomeric region (Fig 2.1). The size of the latter region is dependent on the position of the most distal restriction site for the enzyme(s) used to generate the fragment. Many different restriction enzymes have been used for TRF. Table 2.2 gives an overview of the variety of enzymes and enzyme mixes employed in different studies. A more detailed list can be found in Appendix Table II.I. The size of the non-telomere part of the restriction fragment has been estimated by regression between TL of the same samples measured by TRF and a method that measures only the telomeric repeats (Q-FISH or Q-PCR). Published values range from 2kb to 4.2kb (Hultdin et al 1998; Meeker et al 2002; Cawthon 2002; Ferlicot et al 2003).

To assess the influence of restriction enzymes on TL analysis, several enzymes were tested on a sample pooled from 66 kakapo. Figure 2.3A shows the blot and TL of the same DNA cut with 5 different enzymes and a mix of all of them. TL ranges from 14.68 kb to 17.67 kb with *MseI* and the mix of all enzymes both resulting in 14.68 kb. It has been proposed that birds lack the region of imperfect telomeric repeats (Delany et al 2000), because no Southern blot signal could be detected with probes of telomeric variants (TTGGGG and TGAGGG), but it is not clear if the blot in that study has been denatured or not before hybridization. If not, no signal is expected since all subtelomeric variants are double stranded. *MseI* did reduce the length of the restriction fragment compared to other enzymes in the present study. *MseI* recognizes the

sequence TTAA which is closely related to the telomeric sequence TTAGGG. The reduction of the fragment size indicates that there are restriction sites with an imperfect repeat motive proximal to the telomeres.

Table 2.2: Restriction enzymes used in different TRF studies. Only one study is shown for each enzyme-species combination. A complete list can be found in the Appendix Table II.I.

Enzymes	**Species**	**Reference**
*Alu*I	human	(Norwood & Dimitrov 1998)
*Alu*I	cynomolgus monkey	(Lee et al 2002)
*Hae*III	chicken	(Swanberg & Delany 2003)
*Hae*III	sand martin, dunlin	(Pauliny et al 2006)
*Hae*III	human	(Slagboom et al 1994)
*Hinf*I	mice	(Prowse & Greider 1995)
*Hinf*I	div. birds	(Haussmann et al 2003b)
*Hinf*I	C. elegans	(Joeng et al 2004)
*Hinf*I	human	(Martin-Ruiz et al 2004)
*Hinf*I	frigatebird	(Juola et al 2006)
*Hinf*I	rat	(Zhang et al 2006)
*Hinf*I or *Alu*I	human	(Hastie et al 1990)
*Hinf*I or *Rsa*I	human	(Ishii et al 2006)
*Msp*I	cow, pig	(Kozik et al 1998)
*Taq*I	ginkgo	(Liu et al 2007)
*Alu*I, *Rsa*I	human	(Lahnert 2005)
*Hinf*I, *Alu*I	chicken	(Venkatesan & Price 1998)
*Hinf*I, *Rsa*I	cow	(Lanza et al 2000)
*Hinf*I, *Rsa*I	human	(Grant et al 2001)
*Hinf*I, *Rsa*I	shag, albatross	(Hall et al 2004)
*Hinf*I, *Rsa*I	rat	(Jennings et al 1999)
*Hinf*I, *Rsa*I	pig, cow	(Jeon et al 2005)
*Hinf*I, *Rsa*I	equine	(Argyle et al 2003)
*Hinf*I, *Rsa*I	dog	(McKevitt et al 2002)
*Hinf*I, *Rsa*I	sea urchins	(Francis et al 2006)
*Hinf*I, *Rsa*I	cow	(Ortegon et al 2007)
*Hinf*I, *Rsa*I	Gaddi goat	(Gupta et al 2007)
*Hinf*I, *Rsa*I	goat	(Betts et al 2005)
*Hinf*I, *Rsa*I	dog	(Jang et al 2008)
*Hinf*I, *Rsa*I or *Hph*I, *Mnl*I	human	(Baird et al 2006)
*Hinf*I, *Sau*3AI	pig	(Fradiani et al 2004)
*Msp*I, *Rsa*I	human	(Harley et al 1990)
*Hae*III, *Hha*I, *Hinf*I	human	(Gan et al 2001)
*Hae*III, *Hind*III, *Hinf*I	tree swallow	(Haussmann et al 2005)
*Hae*III, *Hinf*I, *Msp*I	zebra finch	(Haussmann & Vleck 2002)
*Hae*III, *Hind*III, *Hinf*I	alligator	(Scott et al 2006)
*Alu*I, *Cfo*I, *Hae*III, *Hinf*I, *Msp*I, *Rsa*I	human	(Wright et al 1997)
*Alu*I, *Cfo*I, *Hae*III, *Hinf*I, *Msp*I, *Rsa*I	lagomorphs	(Forsyth et al 2005)

One of the often cited disadvantages of TRF is that the fragment length is not only dependent on the actual telomere length, but also on the most distal restriction site for the enzyme(s) used. These restriction sites can differ between individuals, introducing a random error in the measurement.

In the birds investigated here, the use of *Mse*I might minimize this error and increase the over all accuracy of TRF as it cuts closer to the real telomere (Fig. 2.3A). Unfortunately, when tested on 5 different individual kakapo, an extensive banding pattern was observed below the expected telomere smear (Fig. 2.3B). As the bands are detected by the telomeric probe, these fragments consist of a high proportion of telomeric sequence which was flanked by imperfect repeats and probably originate from the border of perfect and imperfect telomere regions. The lack of this signal in the pooled sample (Fig. 2.3A) is probably due to the variability of these fragments between individuals diluting individual banding patterns below the detection limit.

In contrast, restriction with *Hinf*I and *Rsa*I did not show any signal apart from the telomere smear in individual birds tested (Fig. 2.3B). Previous studies in human using *Hph*I and *Mnl*I, which cut the telomere variants TGAGGG and TCAGGG, resulted in an estimated TL of approximately 1 kb less compared to restriction with *Hinf*I and *Rsa*I (Baird et al 2006). In their study no additional banding pattern, as observed in the present study (Fig 2.2B), was found. The reason for that might be that a denaturing blot was used in the present study, so that the probe can bind to fragments resulting from restriction of imperfect telomeric repeats. In contrast, Baird and colleagues (2006) used a probe hybridized to the overhang and therefore only visualised the terminal telomere restriction fragments. A strong correlation between TL estimated with both double digests was observed ($R^2 = 0.987$ (Baird et al 2006, calculated from data presented in Table 1)), suggesting that the influence of subtelomeric regions on TRF using *Rsa*I and *Hinf*I introduces only a low error.

As TRF using radioactively labelled probes was not undertaken in this study, the additional *Mse*I bands (Fig 2.3) could not be avoided. Based on that, and the high correlation

found between TL from enzymes cutting within the imperfect repeat region (*Hph*I/*Mnl*I) and the *Hinf*I/*Rsa*I double digest (Baird et al 2006), *Hinf*I/*Rsa*I digestion was used for further experiments.

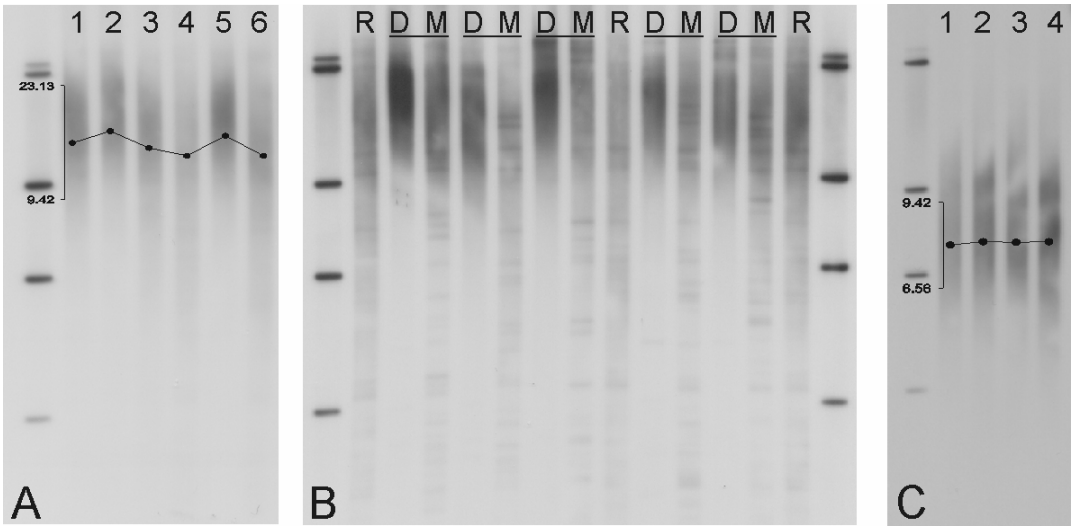

Figure 2.3: Influence of restriction enzymes on telomere length. (**A**) same kakapo DNA restricted with different enzymes, 1: *Rsa*I; 2: *Dpn*II; 3: *Bfa*I; 4: *Mse*I; 5: *Alu*I; 6: all enzymes together. The TL for each lane is overlaid, molecular weight for two bands of weight marker is indicated in kb. (**B**) Five pairs of the same kakapo DNA (underlined) restricted either with *Mse*I (M) or a doubledigest of *Hinf*I/*Rsa*I (D), Note that *Mse*I shows a extensive banding pattern in the lower molecular weight region. R: reference sample. (**C**) same saddleback DNA cut with different concentrations of enzyme-mix *Hinf*I/*Rsa*I, 1+2: 5U each; 3: 20U each; 4: 10U each. The TL for each lane is overlaid, molecular weight for two bands of weight marker is indicated in kb.

Finally, the influence of the concentration of *Hinf*I and *Rsa*I on estimated TL was tested. Using 0.5µg of saddleback DNA in each reaction, telomere distribution and calculated TL did not change for concentrations of 5U, 10U and 20U for each enzyme, indicating that overdigestion does not alter TL estimates (Fig. 2.3C).

2.2.3 Agarose gel electrophoresis

Ensuring even migration of DNA in the agarose gel is important for reproducible results. Careful optimization of conditions like running buffer, percentage of agarose gel, voltage and gel tank properties are necessary. Also, the migration of DNA is not always parallel to the wells. The error that can result from misalignment are shown in Fig. 2.4. The first picture shows a blot

aligned using the molecular weight marker (MWM) on either sides of the gel. The other pictures show the same blot rotated counter clockwise 0.5° and 1°. The TL shown in Fig. 2.4B was calculated using the MWM in the left lane to calibrate the blot.

There was a gradual increase of differences from left to right with approximately 5% deviation between 0° and 1° for two most right lanes (Fig. 2.4C). Note, that the commonly used alignment based on the loading wells would be somewhere between 0.5° and 1°. The measurement error also depends on the TL of the sample, as it is higher for longer telomeres due to the decreasing resolution of agarose gels for high molecular weight DNA. For example, the rotation of 1° in Fig. 2.4A resulted in a shift of the every band of the right MWM of 1.19 cm relative to the left MWM. In the high molecular weight region around 23 kb this distance gets translated in a difference of 6.4 kb whereas in the region around 6 kb it only makes a difference of 1.78 kb.

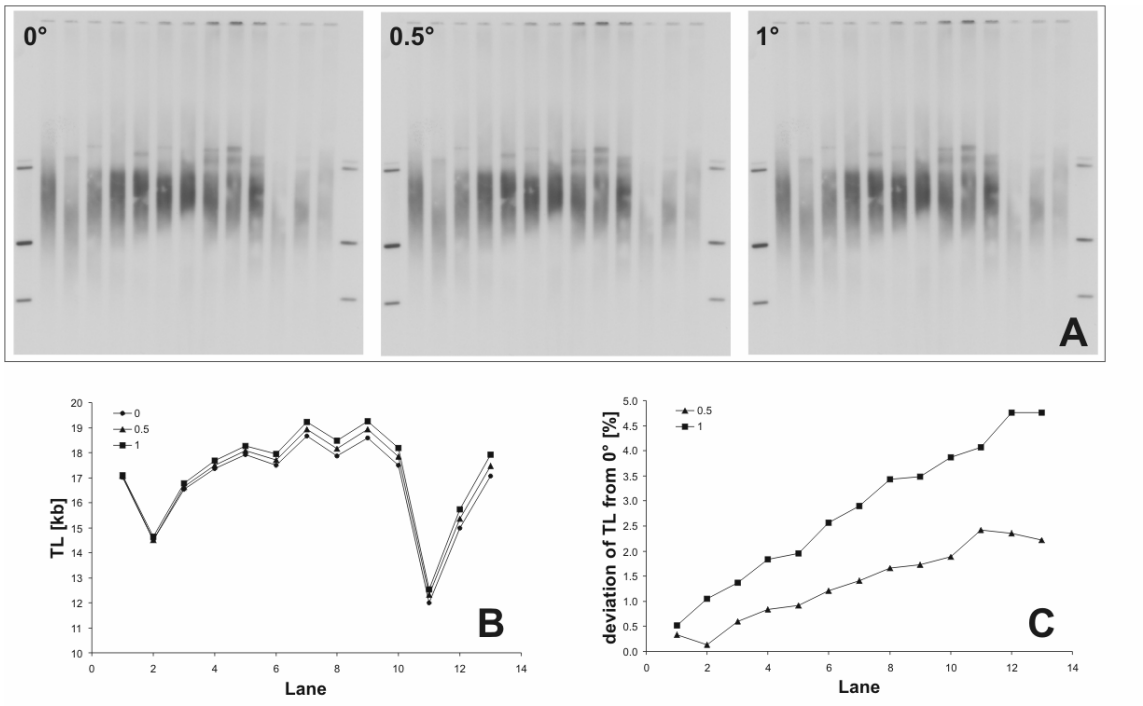

Figure 2.4: Influence of gel alignment on telomere length calculation. (**A**) One blot was rotated 0.5° or 1° counter clockwise and (**B**) TL was calculated from all three pictures. (**C**) Deviation in TL relative to the non-rotated picture for each lane of the gel. Only the left MWM was used for calibration of blot. Note that the samples are unrelated and the lines between the points in (**B**) do not have a biological meaning.

For proper calibration each gel should have at least a MWM on each side of the gel. Additional measures like the inclusion of a reference sample in each gel help to check for reproducibility between blots.

An alternative method has been used by Cawthon (2002). He mixed the restriction fragments with a MWM before electrophoresis. After recording the telomere signal (Fig 2.5A), he stripped the membrane and recorded the position of the MWM in every lane using a different probe (Fig. 2.5B). In this way a precise calibration for each lane is obtained. Fig. 2.5A is a good example of how cryptic uneven migration can be. The TRF smears look straight and parallel to the wells, but a look at the actual migration of the MWM in Fig. 2.5B reveals a increasing distortion of the right lanes.

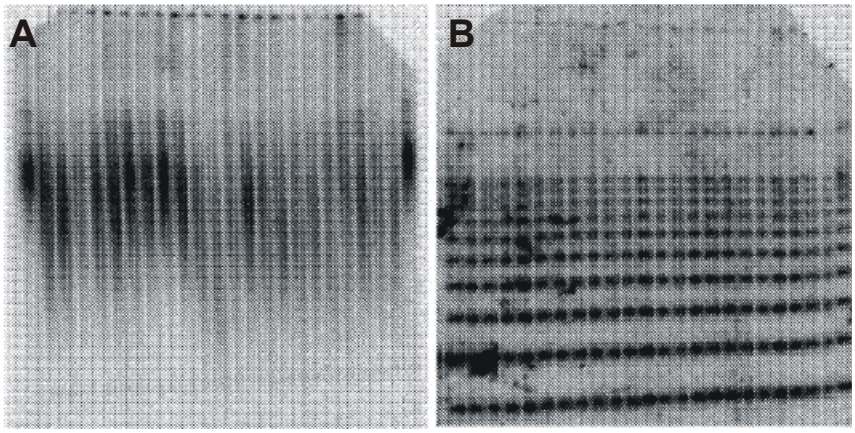

Figure 2.5: Example of uneven migration of DNA. (**A**) Telomere restriction fragments and (**B**) molecular weight marker in the same blot visualized with a different probe after stripping. Note that the uneven migration is not obvious in (**A**). Picture reproduced from Cawthon (2002) with kind permission.

A calibration of each single lane is probably the best way to get reproducible results, but cost of time and money invested to strip the membrane, detect the MWM and calibrate each lane separately can be saved if an even migration is established and controlled for in each blot.

2.2.4 Hybridization and detection

The signal to background ratio of a blot is determined in the hybridization and detection steps. Although optimized protocols are available for different probes and detection systems, mixing different protocols can increase the performance of the experiment. Fig. 2.6 shows two blots hybridized with the same probe in different hybridization solutions. The recommended standard hybridization buffer for Dig-labelled probes (DIG Application Manual for Filter Hybridization, Roche, Fig 2.6A) resulted in a higher background staining and higher intra-gel variation (data not shown) than a modified Church buffer (Church & Gilbert 1984, see appendix I).

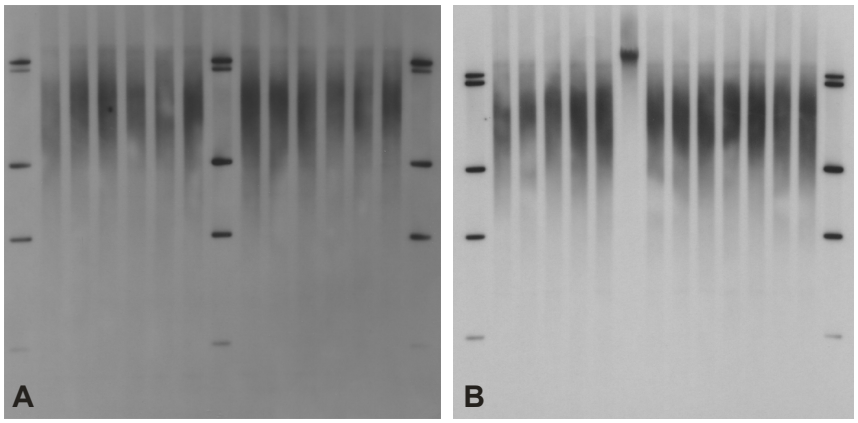

Figure 2.6: Comparison of TRF signal using different hybridization buffer. (**A**) Standard Dig hybridization buffer, (**B**) Church buffer. A lower signal to background ration is obtained with church buffer.

Also, the membrane tended to float in hybridization and washing solutions resulting in temporarily dried out regions across the blot with no or less signal. Lanes tangented by this regions could not be analysed. This problem could be resolved by using paper clips to hold the membrane at the bottom of the tray.

2.2.5 Image analysis

The analysis of the blot and the calculation of mean telomere length is the most diverse and controversial part of the TRF procedure. The descriptions of these methods in publications range from the very precise (Norwood & Dimitrov 1998; Gan et al 2001; Ferlicot et al 2003; Simon et al 2006) to the non existent (Lee et al 2002; Martin-Ruiz et al 2004; Zhai et al 2006), or, are only accessible when reading two additional papers (Counter et al 1994; Prowse & Greider 1995; Henderson et al 1996; Jeanclos et al 2000). Although TRF is claimed to be the only method to measure mean TL in absolute terms (apart from STELA, which is limited to few chromosomes and species) there is no general agreement on how to analyse a TRF blot. The present study shows that different approaches generate not only substantial differences in the magnitude of TL but also in the correlation of TL between samples.

The main factors for analysis of a TFR blot are :

1) background correction
2) calibration of the gel
3) formula used to calculate TL

Because the analysis parameters are all closely related, some of them will be mentioned in this section before they are properly explained. Table 2.3 gives an overview of the different analysis variations tested.

Table 2.3: Overview of different ways to calculate telomere length. OD = optical density, MW = molecular weight, * calculated with telemetric 1.1.1.jar (Grant et al 2001).

Background	Calibration	Formula	Abbreviation
average	exponential	∑(OD•MW)/∑OD	exa
minimum	exponential	∑(OD•MW)/∑OD	exm
average	exponential	∑OD/∑(OD/MW)	exa C*i*
minimum	exponential	∑OD/∑(OD/MW)	exm C*i*
average	exponential	peak of signal	exa P
average	logistic	∑(OD•MW)/∑OD	lga
minimum	logistic	∑(OD•MW)/∑OD	lgm
average	logistic	∑OD/∑(OD/MW)	lga C*i*
minimum	logistic	∑OD/∑(OD/MW)	lgm C*i*
average	logistic	peak of signal	lga P
average	exponential	∑OD/∑(OD/MW)	telom*

The first step to calculate a telomere length is to obtain the distribution of signal intensity against migration. Fig 2.6 already showed an example of a TRF gel. To explain the analysis methods, here the analysis of only one of the 13 sample lanes and the MWM run in the outer lanes of the gel is shown (Fig 2.7A inlay). The remaining 12 lanes were cut out of the picture. The migration was measured inverse as distance from the bottom of the picture. A signal distribution was obtained using ImageJ 1.38 and was plotted against migration (Fig. 2.7). The molecular weight of the marker is indicated in the picture and the graph. Next, the background is subtracted. The recommended method is to define the background level as the intensity of a region of the blot with no specific signal (minimum BG, Fig 2.7B). Note, that the signal distribution ends well above zero at approximately 6cm of migration. Alternatively the average signal of the whole analysis area (including the 12 other samples that are not shown in Fig. 2.7A) is subtracted (average BG, Fig. 2.7C). The remaining graphs are using minimum background to demonstrate the full effect of the calibration models. The migration distance of the MWM is overlaid with an exponential (Fig. 2.7D) or logistic (Fig 2.7E) fit and migration is converted into molecular weight in kb. The TL is then calculated either directly or after converting the signal intensity into relative copy number (Ci, Fig. 2.7F, Equation 2.2) as the weighted mean of the distribution. For details of the formulas see 2.2.5.3.

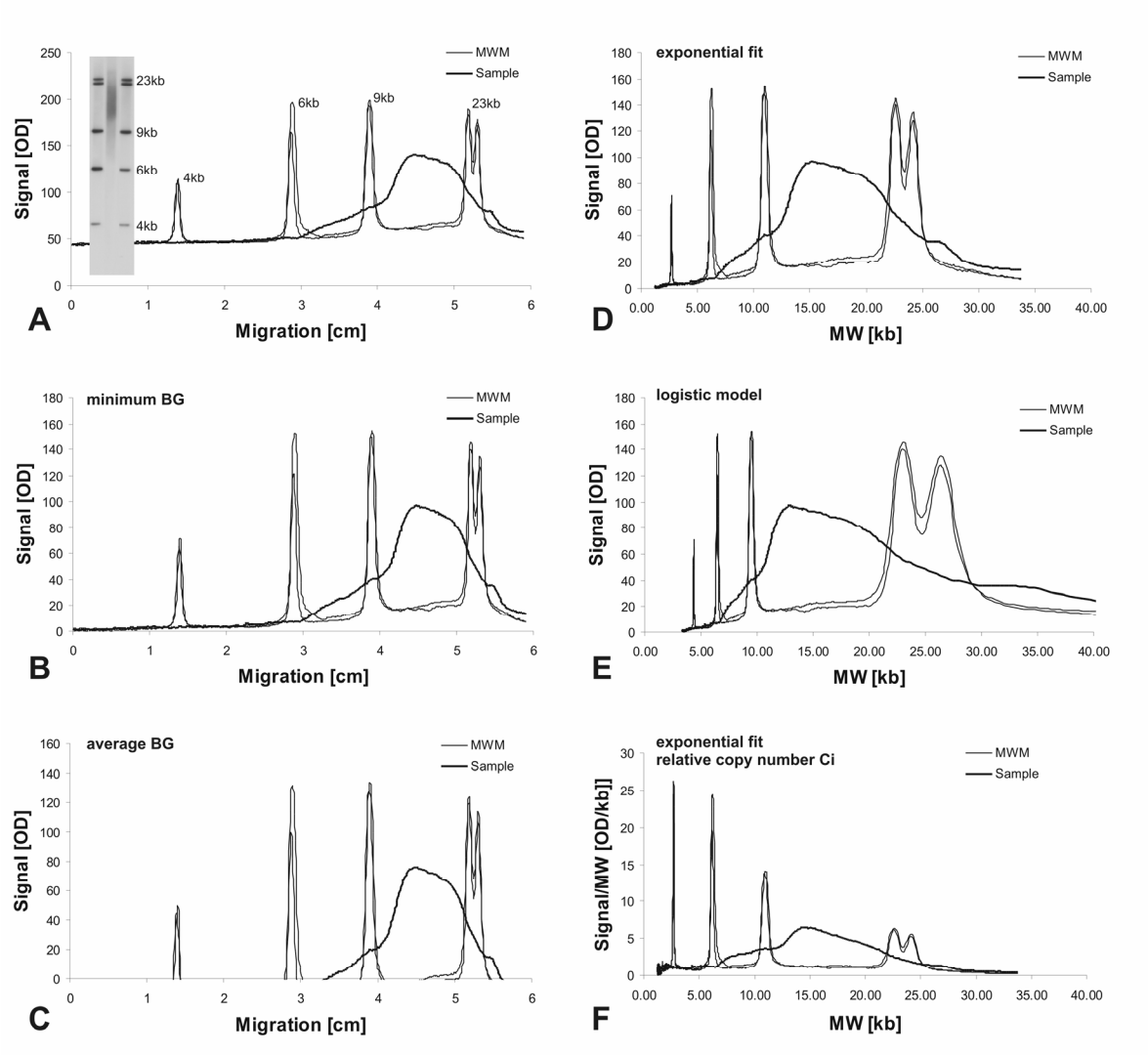

Figure 2.7: Calculation of telomere length. (**A**) The signal of samples and MWM (inlay) was obtained by densitometry and signal intensity was graphed against migration (measured inverse from the bottom of the picture). Minimum (**B**) or average (**C**) background was subtracted and migration converted into molecular weight using exponential fit (**D**) or a logistic model (**E**). TL is calculated as the weighted mean of signal intensity or relative copy number (**F**). Note that migration distance is the distance on the analysed picture and not the real distance on the blot.

One of the main findings of this chapter is that there is no such thing as 'the' telomere length of a sample. A total of 11 different TL for each sample was calculated (Fig. 2.8 and Table 2.3) and it was further tested which measure gives the most reliable and reproducible results. Graphs similar to the one shown in Fig. 2.8 will be used to compare the different analysis forms. Note, that each lane is an independent sample and the lines between the points do not have a biological meaning, but help make the differences between the points more clearly.

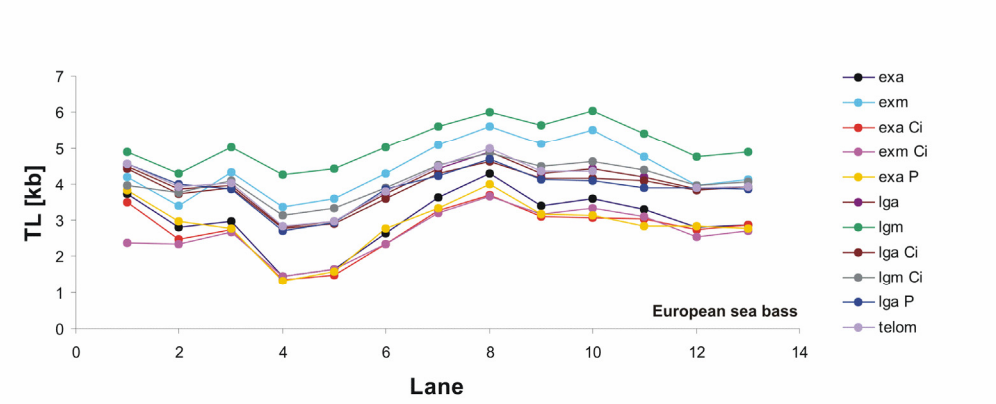

Figure 2.8: Telomere length calculated with 11 different analysis parameters. Each lane represents a independent sample of European sea bass. The lines between the data point do not have a biological meaning. Abbreviations are explained in Table 2.3.

TL in the following sections have been calculated using ImageJ 1.38, CurveExpert 1.38 and MS Excel or telemetric1.1.1.jar.

2.2.5.1 Background correction

Every blot has a unique level of background signal and therefore it is necessary to remove the background in order to compare different blots. It is difficult to find any information about the use of background correction in publications using TRF. Generally a region beside the lanes is chosen that appears representative for the background of the whole blot. This decision is highly subjective and coefficient of variation (CV) of up to 13% have been reported due to misapplied background corrections (Grant et al 2001). Although the subtraction of a representative region outside the lanes seems to be the most intuitive strategy it does not necessarily give the most appropriate results.

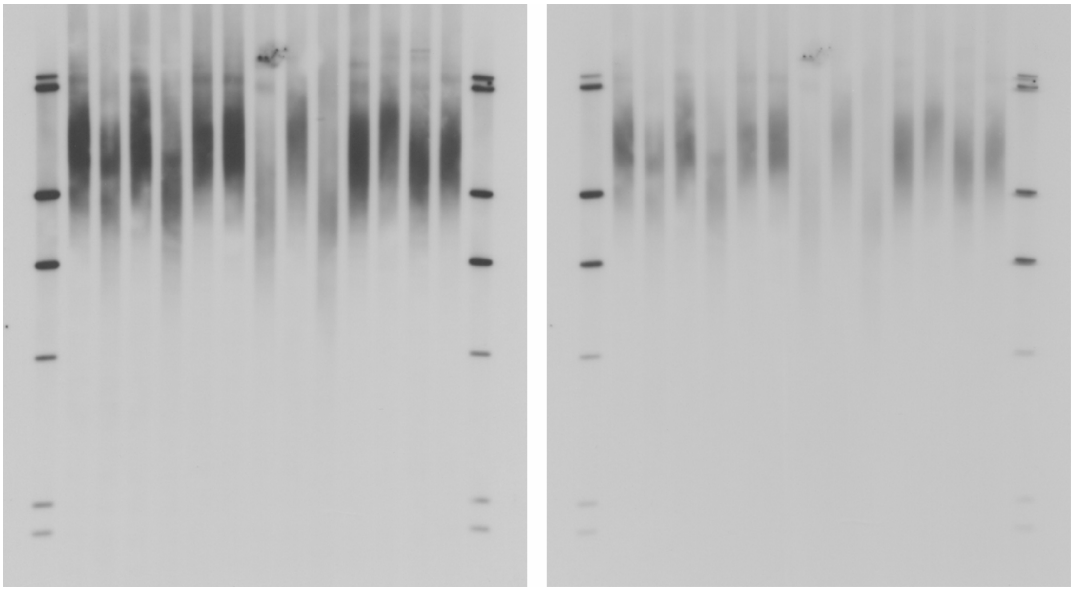

Figure 2.9: Test of background correction in NZ robin. Both pictures are from the same blot, but differ in exposure times. Estimated TL for both blots is shown in Fig. 2.10.

To test the influence of different background correction methods the results of two pictures of the same blot with different exposure times were compared (Fig. 2.9). A good background correction should result in the same TL for the samples in both pictures. To simplify the analysis and be able to automate calculations it was assumed that the background level of a blot would be equal to the lowest signal in any of the analysed lanes (minimum background correction). For test of background correction only TL obtained with exponential calibration of the gel and the formula $\sum(OD_i x MW_i)/\sum OD_i$ (exm and exa, Table 2.3) are presented, because it probably is the most commonly used method, but other calculation methods gave similar results. An example for TL calculated for the same samples from different pictures using minimum background correction (exm) is given in Fig. 2.10. The left graph shows the difference for New Zealand robin representing a species with relative long telomeres and the left graph for European sea bass representing shorter telomeres. On average the TL of a samples differed by 12.58% for NZ robin and 8.22% for European sea bass from the TL of the same sample calculated from the other picture.

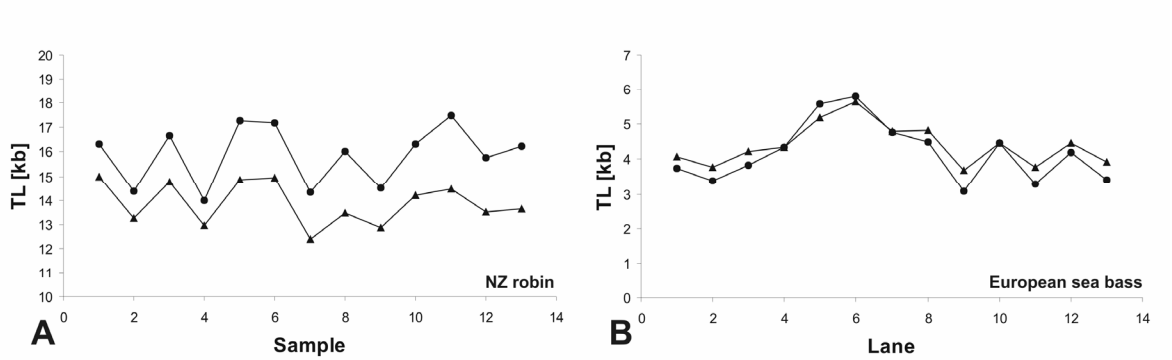

Figure 2.10: Influence of film exposure time on telomere length using minimum background correction. Two pictures of with different exposure times taken from the same blot have been analysed for (**A**) NZ robin and (**B**) European sea bass.

To find the optimal background correction two pictures of the same blot but with different exposure times were taken and several background levels were tested. Starting at the minimum background level, background intensity was increased gradually in 5% steps (Fig. 2.11). IN addition, the average signal of all lanes measured was calculated and used for background correction. An example for two different samples is presented in Fig 2.11. The telomere smear for different exposure times is shown on the right and the calculated TL for different background levels in the graph. There was a high level of variability in the calculated TL depending on the background intensity used for correction. Lower over all signal intensity (i.e. shorter exposure time of the film) resulted mostly but not always in lower TL. Estimated TL of both pictures appeared to approach each other at approximately 15% above minimum background intensity.

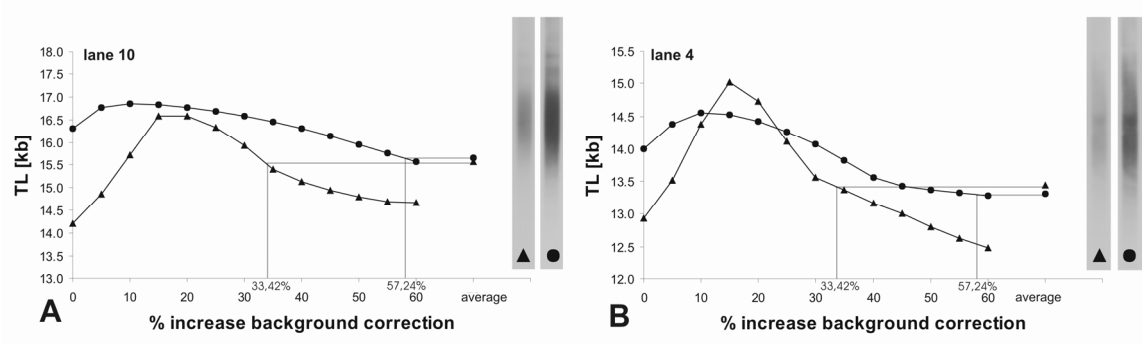

Figure 2.11: Calculated telomere length for different levels of background correction. Two pictures of the same blot were taken with different exposure time. The same lane was than analysed in both pictures (▲ and ●) and TL was calculated for different background corrections. Minimum background level was defined as 0% and TL was calculated for background corrections in intervals of 5%. The average signal intensity from all lanes was also determined and used as background. Average signal equalled 33.42% for picture ▲ and 57.24% for picture ●. The lanes analysed are shown beside the graph for two different NZ robin samples. (**A**) is sample 10 and (**B**) sample 4 in Fig. 2.9. Note that the average background correction shows high similarity of estimated TL for both exposure times although it is based on different background intensities.

Calculation of TL using the average signal intensity also resulted in similar TL for both pictures, despite being based on picture specific background levels (Fig. 2.11). The TL obtained for a constant background level of 15% above minimum and for the average signal from all lanes are shown in Fig. 2.12. Both methods were able to correct the blots for signal intensity and resulted in similar TL for both pictures.

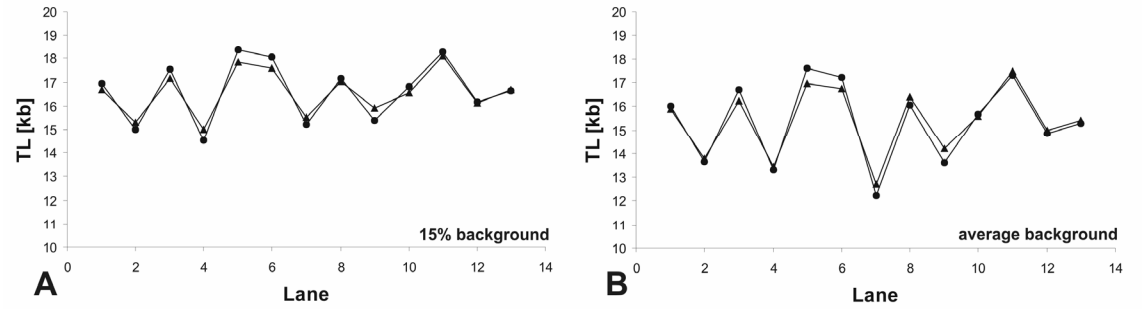

Figure 2.12: TL calculated with different background levels. Background was calculated as a fixed percentage (15%) above the minimum signal intensity (**A**) or as the average of signal intensity of all lanes in the blot (**B**).

The same analysis done for a blot with saddleback DNA showed an optimal percentage for fixed background correction of 35% above minimum background (data not shown). The 15% found for NZ robin did not give equal results for differently exposed pictures. In contrast, background correction based on the average intensity of all samples provided similar TL for the

same saddleback samples with different exposure time. Another advantage of average background correction is that it increases the range of calculated TL. The difference between the longest and the shortest TL of the NZ robin blot was 18.33% for the 15% correction and 27.78% for the average correction. Since there is no such thing as 'the' telomere length of a sample, the method that gives the highest range and therefore the best resolution should be favoured. The average background correction showed also good performance for the remaining analysis variants described in Table 2.3 (Fig. 2.13).

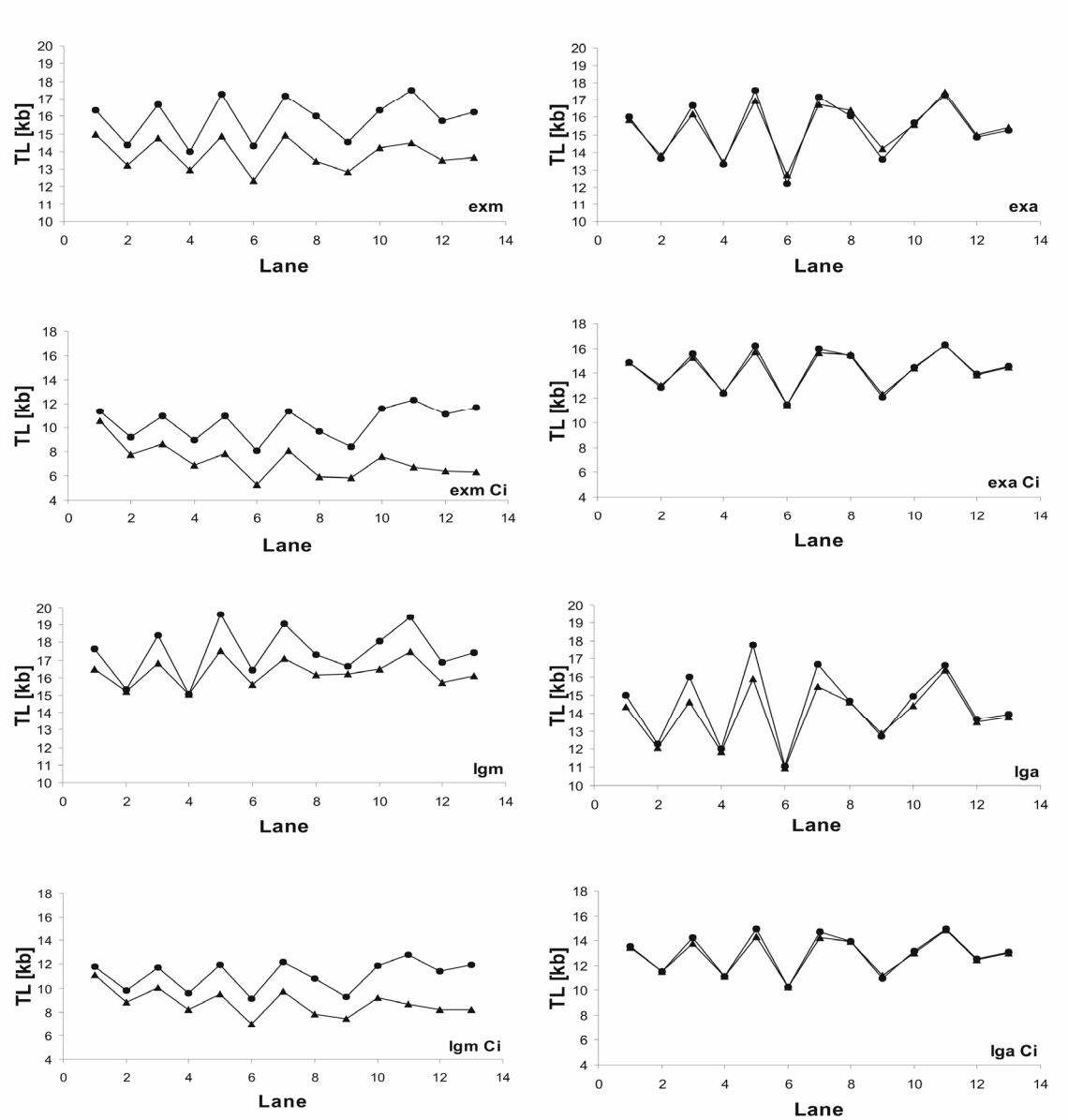

Figure 2.13: Comparison of minimum (left) and average (right) background correction for different analysis methods outlined in Table 2.3. All samples are NZ robin. Abbreviations are explained in Table 2.3.

The minimum background correction showed high deviations between pictures of different exposure times. In contrast, average background correction resulted is an high reproducibility and is easy to use. Based on this results, four of the eleven methods to calculate TL, namely all those based on a minimum background correction, were discarded.

2.2.5.2 Calibration using molecular weight markers

One of the most neglected factors contributing to different TL between publications is the calibration of the gel. Information about the regression or fit used are very rare in TRF publications. Log-linear regression (Haussmann & Mauck 2008) and exponential dependence fit (Norwood & Dimitrov 1998; Feng et al 1999) have been reported, but most papers do not mention the calibration step. In general, the migration of DNA in agarose gels follows a log linear distribution within a defined range (Sambrook & Russell 2001). Within this region the $\log_{10}$ of the molecular weight of a DNA fragment is proportional to the migration distance from the well. The position of the linear range is mainly defined by the agarose concentration of the gel. A log-linear correlation between 500 bp and 15 kb should be achieved with a 0.8% agarose gel and between 700 bp and 25 kb with a 0.5% agarose gel (Sambrook & Russell 2001).

The limiting factor for agarose concentration in Southern blot is the stability of the gel itself. Especially in non-radioactive blotting the gel has to be firm enough to be moved between solutions in the denaturing and neutralization steps. The recommended concentration for TRF is between 0.6% (Telomere Length and TRAP Assay Kit, Pharmingen) and 0.8% (TeloTAGGG Telomere Length Assay, Roche). Both concentrations were tested for calibration in the range from 2kb to 23kb (Fig. 2.14). An exponential fit using CurveExpert 1.38 showed an R^2 of 0.983 for 0.6% agarose and 0.959 for 0.8% agarose.

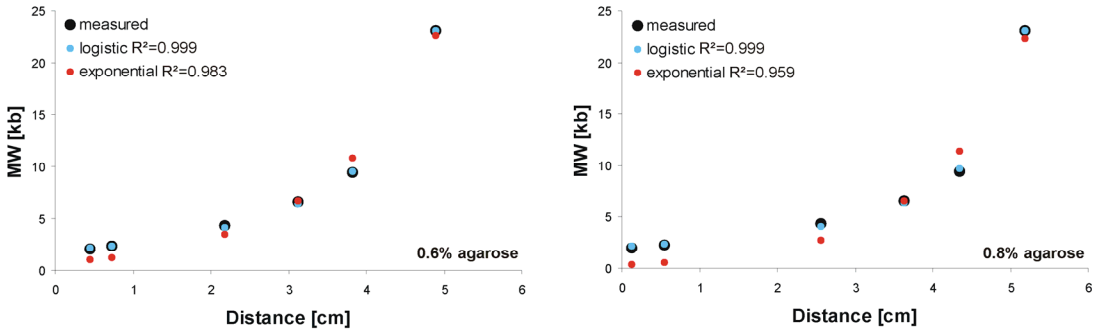

Figure 2.14: Calibration of agarose gels of different concentrations. The migration distances of a molecular weight marker (MWM) was measured from the bottom of the blot and plotted against the molecular weight of the fragments (black). A logistic (blue) and exponential (red) fit is shown for both gels. MWM used was λ *HindIII*.

Interestingly, the exponential regression differed between programmes (here Excel and CurveExpert, Fig. 2.15), introducing another variable when calculating the TL of a sample. Although the higher R^2 favours a concentration of 0.6% agarose, the accompanying disadvantages due to fragility of the gel counteract this advantage.

Using the logistic model following the formula $y=a/(1+b\bullet e^{-cx})$ to calibrate the blot seem to negate any advantage based on gel percentage, resulting in a R^2 of 0.999 for both agarose concentrations (Fig 2.14). It is not clear if this correlation is general or just specific for the electric field in the gel tank used here, but it gave the best correlation coefficient for both agarose concentrations.

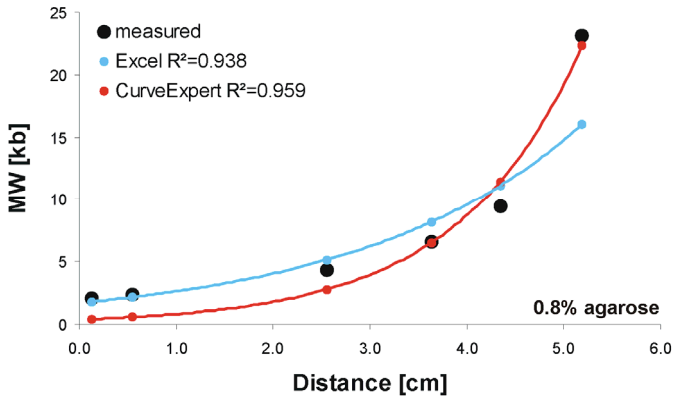

Figure 2.15: Comparison of different programs to calculate an exponential fit. The migration distance of a molecular weight marker (MWM) measured from the bottom of the gel is shown for a 0.8% agarose gel. Exponential fit was calculated with MS Excel and CurveExpert 1.38. MWM used was λ *HindIII*.

In general, the R^2 of the correlation should always be checked and optimized. Unfortunately, some programs (e.g. telemetric (Grant et al 2001)) do not give any indication of the quality of the calibration. A full comparison of the effects of different calibration tactics is presented in section 2.2.5.4 together with the effect of different formulas to calculate TL.

2.2.5.3 Formulas to calculate Telomere length

There are two main formulas for the calculation of TL in literature. The first one is the mean of the distribution calculated by :

$$TL = \sum(OD_i \cdot MW_i)/\sum OD \qquad \text{Equation 2.1}$$

where OD is the optical density at the position *i* and MW is the molecular weight at the same position *i*. This formula assumes that the signal strength is the same for each telomere restriction fragment regardless of its length. Therefore it should be used when the telomere probe is hybridized to the G-rich overhang. The alternative formula uses the optical density adjusted for the size of the restriction fragment by taking the ratio OD/MW. This ratio has been termed relative copy number C*i* (Grant et al 2001). To calculate the mean TL, the OD in equation 2.1 is replaced by C*i*, resulting in

$$\sum(C_i \cdot MW_i)/\sum C_i \quad \leftrightarrow \quad \sum((OD_i/MW_i) \cdot MW_i)/\sum(OD_i/MW_i)$$

$$\leftrightarrow \sum(OD_i)/\sum(OD_i/MW_i) \qquad \text{Equation 2.2}$$

Equation 2 is recommended for denaturing Southern blots, where longer restriction fragments give stronger signals then shorter fragments.

Other calculations have been reported including the position of the peak (Lahnert 2005; Cottliar et al 2006), mean of the positions of the smallest and the largest signal (Jeon et al 2005) or 50% of total OD (Gan et al 2001). It is stunning that a good number of papers do not state how they calculated TL (see Appendix Table II.I). Also, for some publications it was necessary to read two additional papers just to find out how TL was measured and calculated. The next

section shows that this piece of information is critical to conduct any comparison between studies and must not be omitted.

2.2.5.4 Effects of image analysis

The minimum background correction increases the error induces by differences in signal strength and should be avoided (see 2.2.5.1). To investigate the effect of calibration and formula on reproducibility of TL measurement, TRF blots of two species, European sea bass and kakapo, were analysed. In sea bass each sample was measured three times in independent experiments. As the three values come from different gels, the average standard deviation (SD) of all samples equals the inter-gel variation. Furthermore one sample was run four times in the same gel to determine the intra-gel variation. Intra- and inter-gel variations were calculated for the seven remaining analysis methods (Table 2.4). The SD values are not directly comparable because each method has a different range of TL estimates (Fig. 2.16A+B). For example an SD of 100 bp in an assay with a TL range of 2 kb means that 5% of the variation observed is covered by the SD whereas the same SD in an assay with a TL range of 1kb covers already 10% of the variability. To increase the likelihood of detecting a trend in TL change the proportion of the range comprised by measurement error should be as small as possible.

Table 2.4: Validation of different analysis methods for European sea bass TRF. Intra-gel SD was obtained by one sample run four times in the same gel, inter-gel variation is the mean of the SD of all samples (run three times in different gels). Range is the difference between the longest and shortest TL calculated with a given analysis method. Intra- and inter-gel SD divided by the range is a measure of precision of the method (see text and Fig 2.16). Abbreviations are explained in Table 2.3.

Method	Intra-gel SD [bp]	Inter-gel SD [bp]	Range [bp]	Intra/range [%]	Inter/range [%]	Mean [%]
exa	115	180	2608	4.41	6.90	5.66
exa Ci	50	214	2057	2.43	10.40	6.42
exa P	347	344	2664	13.03	12.91	12.97
lga	77	115	1928	3.99	5.96	4.98
lga Ci	50	122	1707	2.93	7.15	5.04
lga P	234	239	2023	11.57	11.81	11.69
telom	99	141	2067	4.79	6.82	5.81

To calculate the proportion of the TL range that is covered by the standard deviation the SD for intra- and inter-gel variation was divided by the range of TL estimates. The difference between SD and the ratio SD/range for inter-gel variation is shown in Fig. 2.16C for different analysis methods. To be able to compare this two measures with different magnitudes (Table 2.4, compare Inter-gel SD and Inter/range) both were normalized against the maximum value in each group. A similar pattern was observes for intra-gel variation. Correction of SD for range (SD/range) did not substantially change the relationship between the error rates of the different methods (Fig. 2.16C). To obtain the mean error proportion for each method, the average of intra- and inter-gel SD/range was calculated (Fig 2.17A, Table 2.4). For sea bass SD/range correlated well with SD for the different methods (Fig. 2.16D), indicating that a larger range of estimated TL coincided with an increase of measurement error.

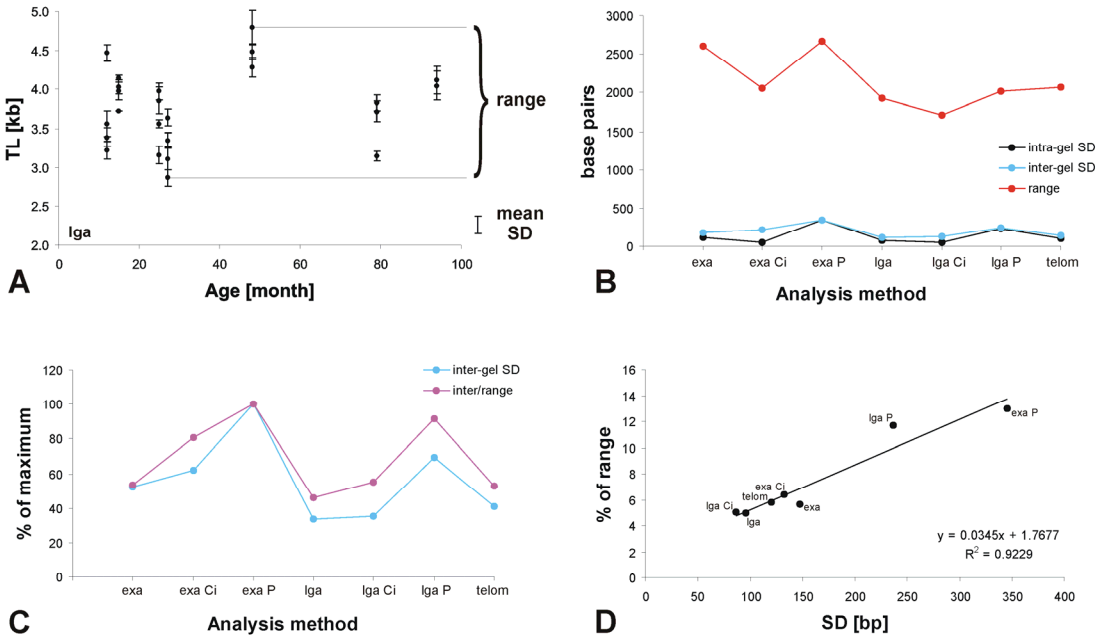

Figure 2.16: Reproducibility of TL estimated by different analysis methods in sea bass (low telomere length). TL of 24 sea bass samples were measured three times on different gels. Inter-gel SD was calculated as the average of all SD and range was defined as the difference between the highest and lowest TL (**A**). Intra-gel SD was obtained by running the same sample four times in one gel (not shown). Inter- and intra-gel SD and range were calculated for each analysis methods (**B**). To obtain a measure of how much of the TL range is covered by the SD, inter- and intra-gel SD were divided by the range. A comparison of SD and SD/range is shown for inter-gel variation (**C**). Values are normalized to the maximum value (100%) of each group. Intra- and inter-gel SD/range were averaged to obtain a mean SD/range for each analysis method (Table 2.4). The relationship between the mean SD/range and the mean of intra- and inter-gel SD is shown in (**D**). Abbreviations are explained in Table 2.3.

The highest mean error rate accounting for 11.69% and 12.97% of the TL range was obtained by the two methods using molecular weight at the peak of the signal distribution (lga P and exa P, Table 2.4 and Fig 2.17A). The other methods showed error rates around 5% to 6.5 % and inter-gel variation was generally higher than intra-gel variation. Methods using correction for multiple binding of probe to longer telomeres (Ci) had a higher difference between inter- and intra-gel variation (Fig 2.17A).

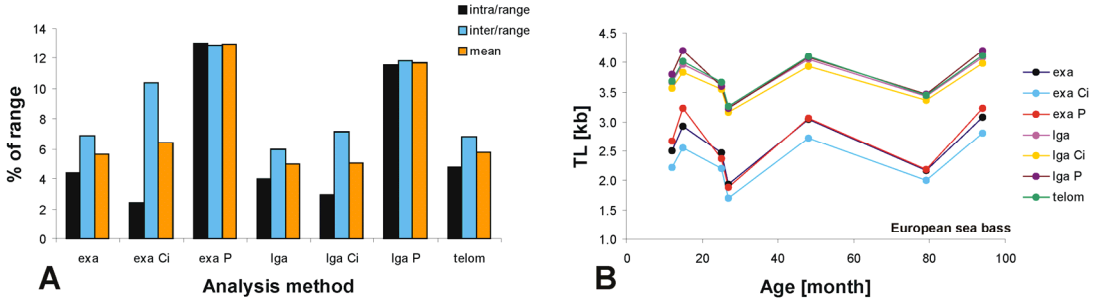

Figure 2.17: Effects of analysis methods on sea bass telomere length. (**A**) Inter- and intra-gel SD/range expressed as percentage of range. The mean of both SD/range represents the proportion of the range of measured TL that is covered by inter- and intra-gel variation. (**B**) TL against age classes demonstrating the influence of analysis method on TL estimate. Mean TL of all age classes differs significantly between exponential (ex) and logistic (lg) calibration (Students t-test, $p<0.001$). Abbreviations are explained in Table 2.3.

The mean TL for each age class of sea bass is shown in Fig. 2.17B. All analysis methods revealed a similar pattern between these classes, but the magnitude of the estimated TL was significantly different between the methods using exponential (ex) or logistic (lg) calibration of the gel. Interestingly, the TL calculated with telemetric, which uses an exponential fit, clustered with the methods using a logistic model. This suggests that telemetric uses a different algorism to find the best fit curve than the programme used in this study to find the exponential fit (curveExpert 1.3).

The influence of analysis method on the results obtained for kakapo, a species with relatively long TL, was also investigated to provide a counterpoint to the sea bass, which have short telomeres. Due to the number of samples used (n = 68) it was not possible to run each

sample three times. Instead, one sample was measured in triplicate on each blot to obtain the intra- and inter-gel variation (Table 2.5).

Table 2.5: Validation of different analysis methods for kakapo TRF. Intra- and inter gel standard deviation was obtained from one sample ran in triplicates on each gel. The range of TL is the different between the longest and shortest TL calculated with a given analysis method. Abbreviations are explained in Table 2.3.

Method	Intra-gel SD [bp]	Inter-gel SD [bp]	Range [bp]	Intra/range [%]	Inter/range [%]	Mean [%]
exa	460	492	4185	10.99	11.77	11.37
exa Ci	497	641	4373	11.37	14.66	13.01
exa P	1177	760	6622	17.77	11.48	14.63
lga	616	485	5595	11.01	8.67	9.84
lga Ci	496	623	4523	10.97	13.78	12.37
lga P	1335	858	7621	17.52	11.26	14.39
telom	485	467	4610	10.52	10.13	10.33

The intra- and inter-gel SD and the range for kakapo are shown in Fig 2.18A. In contrast to sea bass there was a difference in the pattern of different methods for SD and SD/range in kakapo (Fig 2.18B). The methods with the highest SD (exa P and lga P) did not have the highest SD/range, because they showed a greater range of measured TL (Fig. 2.18A, Table 2.5). However, the intra-gel SD/range variation for these two methods was very high compared to the inter-gel SD/range, so that the mean SD/range for intra- and inter-gel variation exceeded the other methods (Fig. 2.18C and Table 2.5).

The proportion of range covered by the measurement error was more than double for kakapo compared to sea bass, with the exception of peak based methods (P) that were already high in sea bass (Fig 2.18C). The correlation between SD and SD/range was less pronounced (compare Fig 2.16D and Fig 2.18C), indicating a stronger influence of TL range for measurement in species with longer telomeres.

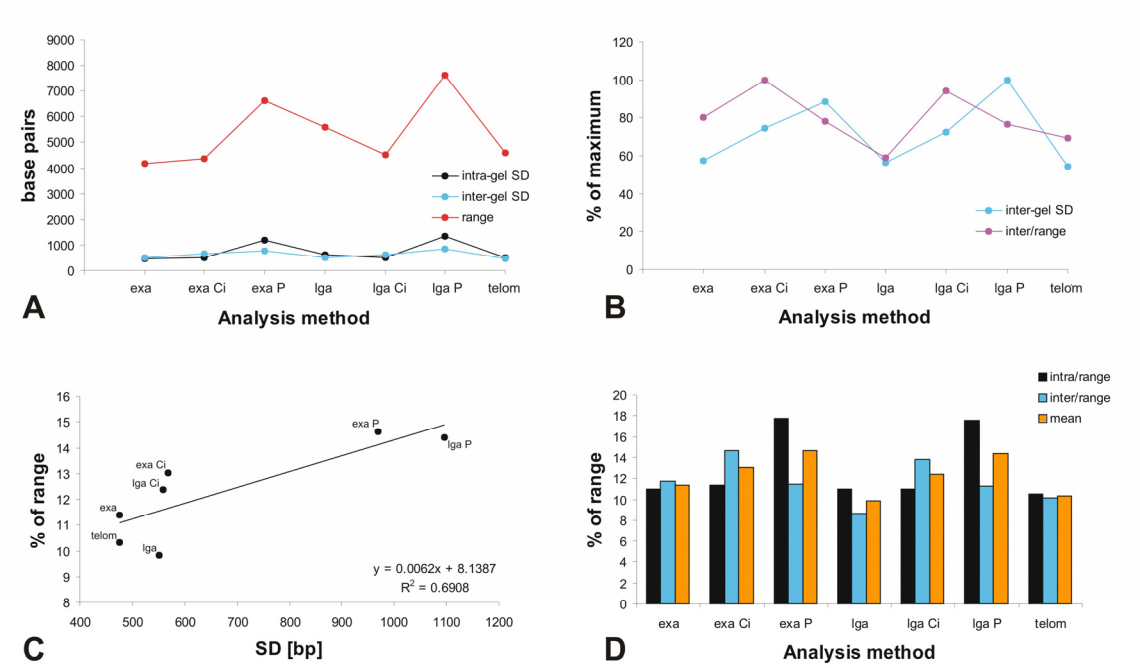

Figure 2.18: Reproducibility of TL measured by different analysis methods in kakapo (high telomere length). TL of one kakapo reference samples was measured three times on each blot to obtain intra and inter-gel SD (**A**). The range was calculated from 68 samples measured beside the reference sample. (**B**) Comparison of SD and SD/range for inter-gel variation. Values are normalized to the maximum value (100%) of each group. (**C**) Relationship between mean SD/range and the mean of intra- and inter-gel SD. (**D**) Inter- and intra-gel SD/range expressed as percentage of range. Abbreviations are explained in Table 2.3.

Again, the methods using the peak of the signal distribution (P) showed the highest error (Fig. 2.18D). The methods using signal intensities corrected for multiple binding (Ci) had a higher error than the methods not correcting for that. The influence of analysis method on the whole dataset is shown in Fig. 2.19. The mean TL of all samples varied from 14.47 kb for lga Ci to 17.96 for exa P.

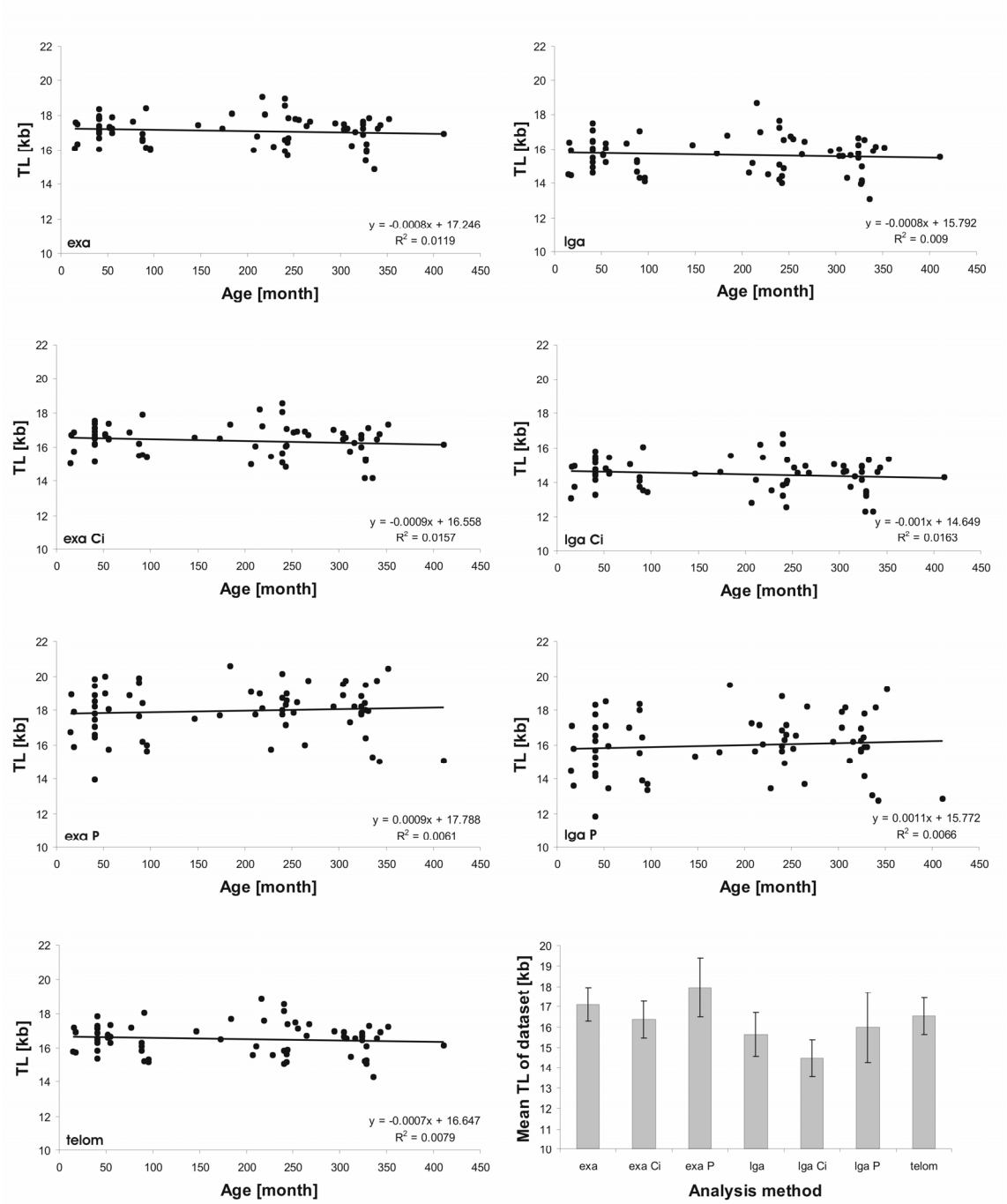

Figure 2.19: Effects of analysis method on data trends in a population. TL was calculated for 68 kakapo using seven different methods. Mean TL of all samples varies from 14.47 kb for lga Ci to 17.96 for exa P. Abbreviations are explained in Table 2.3.

Unfortunately, neither sea bass nor kakapo showed a correlation between TL and age. To investigate the effect of analysis method on estimated telomere rate of change (TROC), TL was measured for New Zealand robin and saddleback (Fig 2.20), because samples from chicks and very old birds were available for these species. The two age groups should represent the

maximum and minimum TL within these species (see Chapter 4). Telomere length data were obtained from the five youngest and the five oldest birds available and measured on one blot for each species. The regression line indicates that telomere length changes depending on age with the slope indicating the theoretical monthly loss of telomeric DNA. For both species slope changes for different analysis methods, and for saddleback the coefficient of determination (R^2) also alters based on analysis methods.

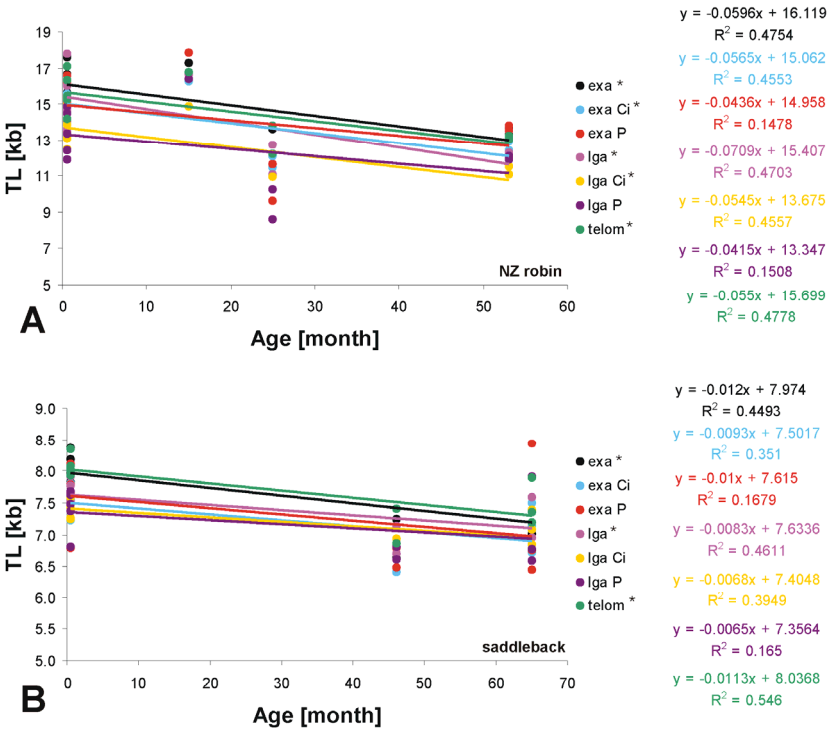

Figure 2.20: Influence of analysis method on telomere rate of change (TROC). The decline of TL with age is shown for NZ robin (**A**) and saddleback (**B**). The slope of the regression line equals the monthly loss of telomeric DNA in kb and the coefficient of determination (R^2) indicates the proportion of TL that can be explained by age. Significant correlation between age TL and age is indicated by * (linear regression, $p<0.05$). Abbreviations are explained in Table 2.3.

Due to the small sample size, the regressions might not fully reflect the dynamics for the whole populations, but nonetheless the differences in slope and coefficient of determination R^2 show how the analysis method can influence the estimate of such important parameters like rate of telomere loss and proportion of loss explained by age (R^2).

Both methods that use the peak of the signal curve to determine the TL of a sample had exceptional high SD and SD/range in sea bass (Fig 2.17A) and still elevated values in kakapo

(Fig 2.18D). Furthermore they failed to detect any correlation between TL and age in NZ robin and saddleback and had a low coefficient of determination (Fig 2.20). A possible explanation is that Southern blots always have small fluctuations of signal intensity across a blot due to extensive handling. If the signal of a smear shows a plateau like pattern rather than a peak, this fluctuations can substantially shift the calculated TL because it only depends on one pixel (the maximum). In contrast, the other methods use a weighted mean which is much more robust against small fluctuations. Apart from the high error rate this method has also a biological incongruity. It assumes that the peak of the distribution shifts in proportion to the mean telomere length. It has been shown the rate of telomere decline is not the same between telomeres of different length (Teixeira et al 2004), resulting in a change of the shape of the distribution, including the peak.

The remaining methods showed a two fold increase of SD/range from sea bass to kakapo. This is probably due to the decrease of agarose gel resolution with molecular weight making longer telomeres more prone to measurement errors. Correcting for multiple binding of probe (Ci) generally resulted in higher SD/range due to intra- and inter-gel variation in kakapo (Fig 2.18D), but mainly inter-gel variation in sea bass (Fig. 2.17A). There seems to be some feature of this method that makes it very precise within gels, but less precise between gels when measuring small telomeres. This trend was not observed in kakapo with long telomeres. Although the SD of the Ci methods were mostly smaller than the SD of non-correcting methods, they had a higher SD/range. The lower error was only due to a lower resolution (range) of the methods. The logistic model calibration without multiple binding correction (lga) showed the lowest error on both dataset, probably due to the better fit to the MWM lanes as shown in 2.2.5.2. Interestingly, the telometric programme, that uses exponential fit and correction for multiple binding, also produced low error rates that lay somewhere between lga and exa.

The estimated telomere length differed between the two calibration models. In sea bass there was a significant difference between logistic model (lg) based and exponential fit (ex)

methods (students t-test, $p<0.01$). The mean TL of all samples was about 1.5kb or 40% lower in ex than in lg. Telometric estimated TL similar to the logistic model. Conversely, ex gave higher TL than lg for kakapo (around 1,5kb or 10%) and telemetric estimates were more in the range of exponential fit. These differences emphasise the importance of calibration models when TL is compared in different studies. Although only few authors mention calibration formulas, the exponential fit is probably used most often, because DNA migration is supposed to be proportional to the logarithm of its molecular weight (Sambrook & Russell 2001). Problems arise when different programmes are used to calculate the fit. Most scientific graphical programs, like telemetric, do not give any indication of the goodness of fit. Also, as shown in 2.2.5.2. different programs calculate quite different equations for the exponential fit.

A slight difference could also be observed with and without correction for multiple binding. Ci analysis tend to estimate lower TL (Fig 2.17B and Fig. 2.20). An overestimation of TL by telemetric as reported by (Haussmann & Mauck 2008) could not be found, but the authors used non-denaturing radioactive Southern blot and telemetric version 1.2, which can be run without multiple binding correction.

For the estimation of age based on telomere length it is important to find the method that has the highest ability to detect change in telomere length. This was tested on small datasets of NZ robin and saddleback (Fig 2.20). To eliminate intra-gel variation only one blot containing the five youngest and five oldest birds available was analysed. The correlations obtained are just approximations and the differences between the methods might decrease if more individuals are measured, but nevertheless arise from the same set of raw data. In both species the peak methods (p) fail to detect a correlation between TL and age. Ci methods showed a significant correlation in NZ robin but not in saddleback, where the R^2 was also reduced. Exa, lga and telemetric were all able to detect a significant correlation and age could explain about 50% of the variation of TL (R^2 between 0.45 and 0.55). However, high variation in the telomere rate of change (TROC) was observed between these three methods. NZ robin telomeres decreased between 660 bp/year

(telemetric) and 851 bp/year (lga) and saddleback telomeres between 100 bp/year (lga) and 144 bp/year (exa). Due to the small sample size we can not draw conclusions from the TROC, but the high variability obtained from the same raw data emphasised the influence of analysis methods on the most important variable for molecular ageing in animals (TROC).

The differences in error rate, magnitudes of TL values and ability to detect correlations might seem marginal, but as discussed in previous sections, error sources are numerous in TRF and each improvement is important. The use of specialized programs like telemetric standardizes the calculation of telomere length, but on the other hand the user has no control over the actual calculations. The way of obtaining the exponential fit is unknown and the goodness of fit is not given. For the sake of comparability to other studies and exponential fit seems to be the most appropriate way, but as different programmes estimate different regression equations the comparability is already widely lost. The logistic model calibration without correction for multiple binding (lga) was chosen for all further analysis of the present study, because it showed the lowest error rate in terms of intra- and inter-gel variation adjusted for range and was able to detect a correlation between TL and age in NZ robin and saddleback.

2.2.5.5 Telomere optimal estimate (TOE)

Recently, a new analysis method, the telomere optimal estimate (TOE), has been proposed to generate TRF results of higher quality and more objectivity (Haussmann & Mauck 2008). The key factor for this new approach is the analysis window. Unlike the traditional analysis that measured the whole telomere smear, only a small part of the telomere signal (16% - 57% of the telomere distribution in the examples given) is analysed. This subset is supposed to be of higher biological relevance than the whole telomere distribution (Haussmann & Mauck 2008).

The first step to estimate the telomere optimal estimate (TOE) window is to obtain the average signal distribution of the four oldest and the four youngest individuals available (Fig

2.21). Due to the decrease of telomere length, the distribution of the old individuals should show a shift to the left (lower MW) compared to the younger individuals. Moving from the left side of the graph (bottom of the picture) the first point where both distributions meet is determined and called the telomere intersection point 1 (TIP1, Fig. 2.21). Moving right along, the next intersection is called TIP2. The geometrical middle (in cm) is the telomere optimal estimate (TOE) and the TOE window spans from the bottom of the gel (0cm) to the TOE (Fig. 2.21). If only one TIP exists then the TIP equals TOE. Haussmann & Mauck (2008) found the best correlation between TL and age near this analysis window.

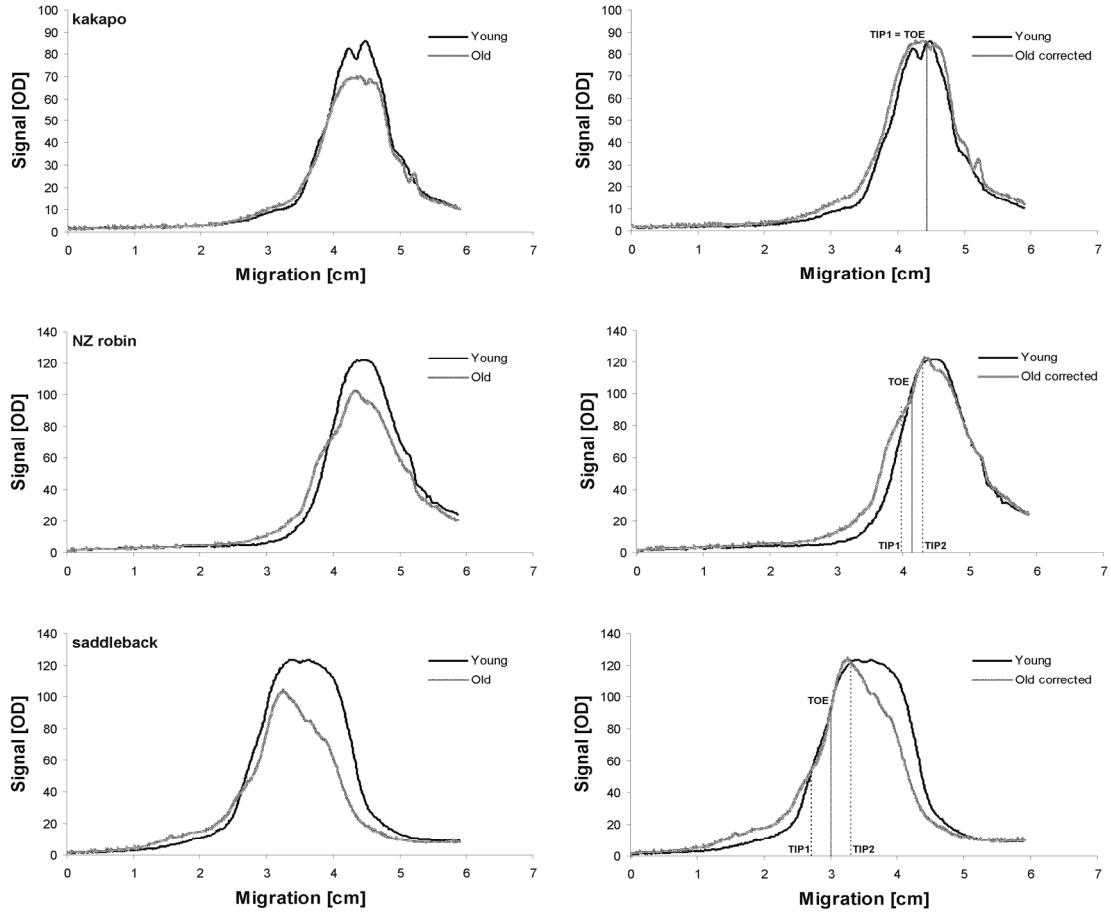

Figure 2.21: Calculation of telomere intersection point (TIP) and telomere optimal estimate (TOE). (**left**) Average signal distribution of the five youngest and the five oldest birds of three different species. (**right**) Distributions corrected for maximum signal intensity and positions of TIP and TOE.

The TOE method was tested for three species: kakapo, NZ robin and saddleback. The first problem encountered was that the signal distributions did not show the same intensities. As

shown in Fig. 2.21 the intensity of the distribution of older birds was lower in all species. Because changes in intensity have a huge influence on the position of TIP and TOE, some standardisation had to be introduced. Therefore, the distribution for old birds was normalized to the level of the young birds by calculating the ratio of the maximum TL of young and old and multiplying each point of the old birds distribution with the obtained ratio (Fig. 2.21). Then, TIP and TOE could be determined and the telomere length TL for the TOE window could be calculated. The regression between TL and age is shown in Fig. 2.22 for the whole telomere smear and the TOE window.

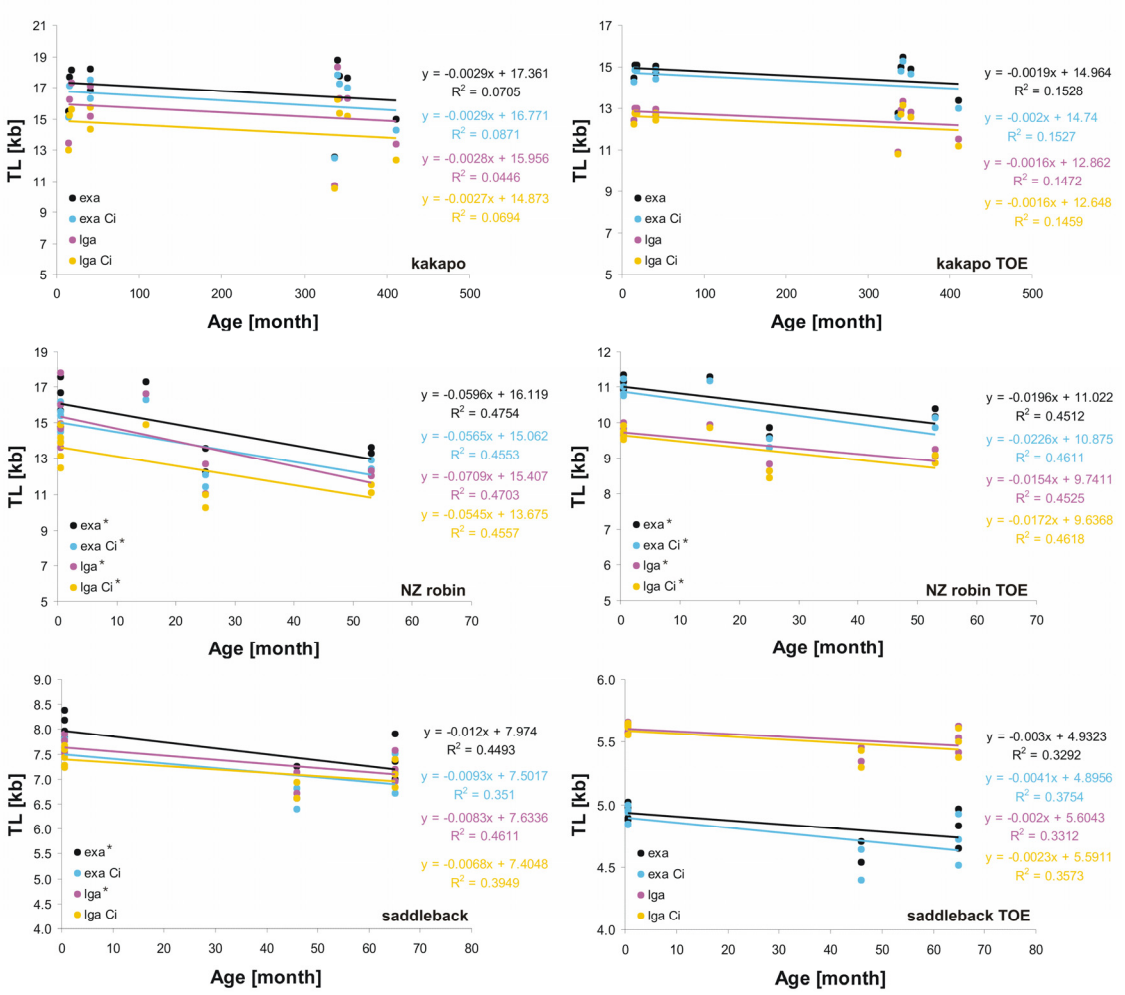

Figure 2.22: Correlation between telomere length and age for three different species calculated from the whole smear (**left**) or the TOE window (**right**). Significant correlations between age TL and age is indicated by * (linear regression, $p<0.05$).

Although the TOE window showed a slightly higher R^2 for kakapo, there was still no significant correlation between TL and age. For NZ robin both methods resulted in similar R^2 and for saddleback the R^2 of lga decreased from 0.46 for the whole smear to 0.33 for TOE, accompanied by a loss of significance. The rate of telomere loss calculated with the TOE window was about half the rate from the whole distribution in kakapo and 4-5 fold less for NZ robin and saddleback (Fig. 2.22 and Table 2.6).

Table 2.6: Comparison of telomere analysis using whole distribution and TOE window. Telomere loss rate and coefficient of determination (R^2) is given for the regressions in Fig 2.22. All values are calculated using lga analysis. Significant correlations between age TL and age are indicated by * (linear regression, $p<0,05$).

Species	**Whole distribution**		**TOE window**	
	Telomere loss [bp/year]	**R^2**	**Telomere loss [bp/year]**	**R^2**
kakapo	33.6	0.05	19.2	0.15
NZ robin	850.8	0.47 *	184.8	0.45 *
saddleback	99.6	0.46 *	24	0.33

The TOE method did not show any advantage over the analysis of the whole telomere distribution. This could result from a variety of different reasons:

- The position of the TIP's and consequently the TOE are highly dependent on the signal intensity of the two distributions. In the blots obtained in the present study the signal strength for young and old birds was not similar. The exact reason for this is not known. All samples have been quantified and the same amount of DNA has been loaded in each well. The length difference between the age groups could account for some variation as in a denaturing blot more probe can bind to longer telomeres so that the total signal intensity is higher for birds with longer telomeres. On the other hand there was no difference in the telomere distribution (i.e. length of telomeres) between young and old birds in kakapo, but still a difference in signal intensity between young and old birds (Fig. 2.21). The TOE method was developed using in-gel hybridization

and a radioactively labelled probe hybridized only to the overhang. In this way each telomere should have the same signal intensity regardless of its length. The correction used ion the present study (multiplying each point of the old bird distribution with the ratio of both) to obtain the same signal strength for both age classes could have distorted the real distribution and the positions of TIP and TOE.

- Alternatively the TOE method is not as powerful as it is claimed to be. Several questions remain unsolved. The distributions presented by Haussmann & Mauck (2008) show a shift of the telomere signal between the age classes from higher to lower molecular weight (MW) (Haussmann & Mauck 2008), consistent with at least the saddleback distributions in the present study (Fig. 2.21). What is not clear is why the change of TL should be easier to measure if half of the shift is ignored. Shouldn't the change be more pronounced if both regions, the one that gains signal (low MW) and the one that looses signal (high MW), are included in the analysis? In addition Haussmann & Mauck (2008) found no difference between age classes in the distribution of telomeres that are above the second TIP. Consequently, if they are the same they should not influence the result if included in the analysis. Unfortunately, no information about the actual molecular weight of the TIP and TOE regions was provided in the Haussmann & Mauck study. It is mentioned that pulse field gel electrophoresis (PFGE) was used to reveal the smallest telomere fragments. PFGE is advantageous over constant field electrophoresis in that it resolve DNA fragments of more than 30kb. That implies that fragments up to at least 30kb are included in the TOE window. This contradicts the presented telomere length of 2-5kb (Haussmann & Mauck 2008).

I suspect that the TOE window method only corrects for the lack of resolution of gel electrophoresis for high molecular weight. The gel shown by Haussmann & Mauck (2008) is of

bad quality in respect of resolution (spreading of the MWM) and sharpness, thus the calibration using the MWM shown leaves room for interpretation. As discusses before, small differences between lanes or the rotation of the picture can result in huge differences in TL in regions of 20+ kb, which show the main telomere signal in Haussmann & Mauck (2008). These differences can easily be the cause for the lack of correlation observed when using the whole telomere distribution. In this case all that TOE analysis does is ignoring the measurement error that is pronounced in high molecular weight regions. A proper optimization of TRF would therefore result in similar, if not more reliable results.

A meta-analysis of TL change in birds is significantly hindered by the lack of information given on the analysis method used in a number of publications. Several publications have been published using TOE window without stating it in the method section (Haussmann, personal communication). The TOE analysis assumes telomere shortening with age and identifies the region that shows the best correlation between TL and age, an approach that might be adequate for ageing birds, but does not allow comparison of TL or TROC with studies using the whole telomere distribution. As shown above, this method yields significantly different TL and TROC (both up to 5 fold difference, see Haussmann & Mauck 2008 and Fig 2.22). To maintain comparability between publications and integrity of the scientific information content, if TOE is used, this information must not be omitted from any publication.

2.3 Telomere classes in birds

The distribution of telomeres in avian species has been controversial. While one group found abundant interstitial telomeric repeats and telomeres up to approximately 25 kb in chicken (Venkatesan & Price 1998), another group reported additional ultra long telomeres (up to 2 Mb) in chicken and other bird species (Delany et al 2000). Unfortunately this discrepancy and different findings in telomerase activity lead to unjustified criticism by misciting results (Delany

et al 2000; Taylor & Delany 2000). In terms of maximum telomere length, the most probable explanation for the discrepancy is a difference in the electrophoresis protocol.

However, TRF fragments have been divided into three classes (Delany et al 2000). Class I consists of distinct bands in the range of 0.5 kb to 8-10 kb, that are only detected in denaturing gel/blots and resistant to *Bal*31 digestion (Venkatesan & Price 1998; Delany et al 2000, but not supported by the presented figure), suggesting an interstitial position in the chromosomes. Class II telomere fragments have a size range of 8-10 kb to 30-40 kb and show a decrease of length with age or population doubling in cell culture. These fragments shorten with increasing time of *Bal*31 digestion and are thought of as similar to human telomeres, as they form a classical smear in TRF. Class III includes all fragments >30-40 kb. Although a digestion with *Bal*31 showed a fading of signal rather than a shift towards lower molecular weight (Delany et al 2000), they have been suggested to be real terminal telomeres, but it is not known if they shorten with age. These categories are deduced from chicken only and rather subjective in respect to their borders.

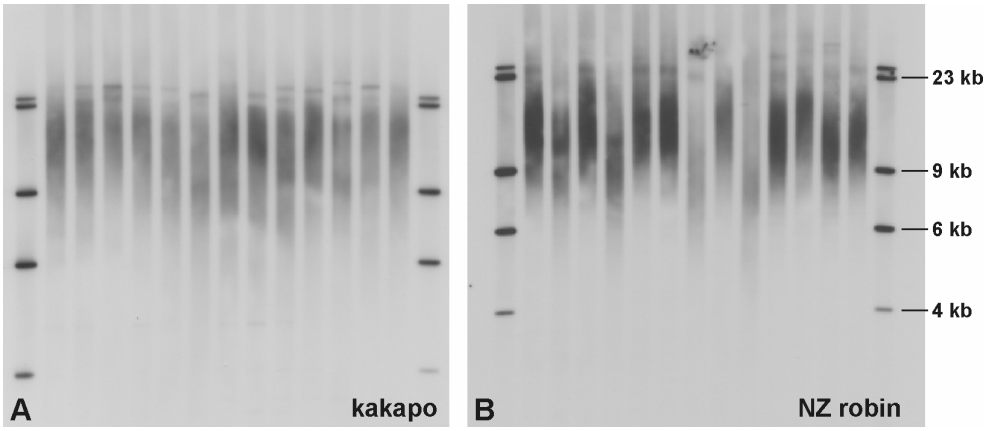

Figure 2.23: High molecular weight bands of approximately 20-30 kb in kakapo and NZ robin. Note that bands occur at different length, indicating that they are not larger than the limit of mobility of conventional electrophoresis. Molecular weight is indicated on the right.

In the present study, class III fragments were only present in zebra finch (Fig. 2.26) and the class II fragments extended well below 8 kb in some species. However, some distinct bands in the 20-30 kb range were visible in kakapo and, very faint, in NZ robin (Fig 2.23). Constant field electrophoresis is not able to resolve high molecular weight bands above approximately

30kb (Haussmann & Mauck 2008). All larger fragments migrate at the same speed. In contrast, the high molecular weight fragments observed in kakapo and NZ robin displayed different lengths, indicating that they are still resolvable under the electrophoresis conditions used. Accordingly they should be smaller than approximately 30kb and therefore Class II telomere fragments. It is not clear if these fragments are real telomeres or even large fragments of interstitial repeats. Their low signal intensity in NZ robin suggest a mixed composition of telomeric repeats with other sequences. Using the background correction described in 2.2.4.1, these fragments did not contribute to the analysed telomere signal in NZ robin.

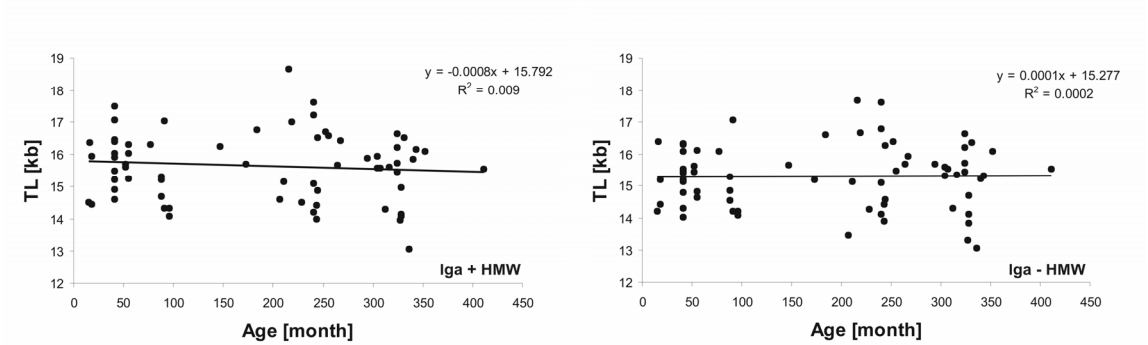

Figure 2.24: Distribution of telomere length against age in kakapo with (**A**) and without (**B**) inclusion of high molecular weight bands. No correlation was observed in either of the distributions (linear regression)

To test the influence of the 20-30 kb bands on TL estimation in kakapo, they were removed manually from the signal distribution of each samples. This correction showed little effect on the distribution of TL against age. Some points got shifted towards lower TL, but still no correlation with age could be detected (Fig 2.24).

Class I telomeric repeats were abundant in all species, but did not interfere with class II fragment measurement in any species apart from zebra finch. The two factors influenced the detection of these interstitial repeats, the used restriction enzymes and the exposure time of the film. The effect of restriction enzymes has already been shown in Fig. 2.3 (section 2.2.2). The use of *Rsa*I and *Hinf*I did not result in any detection of interstitial repeats at the exposure times used for TRF.

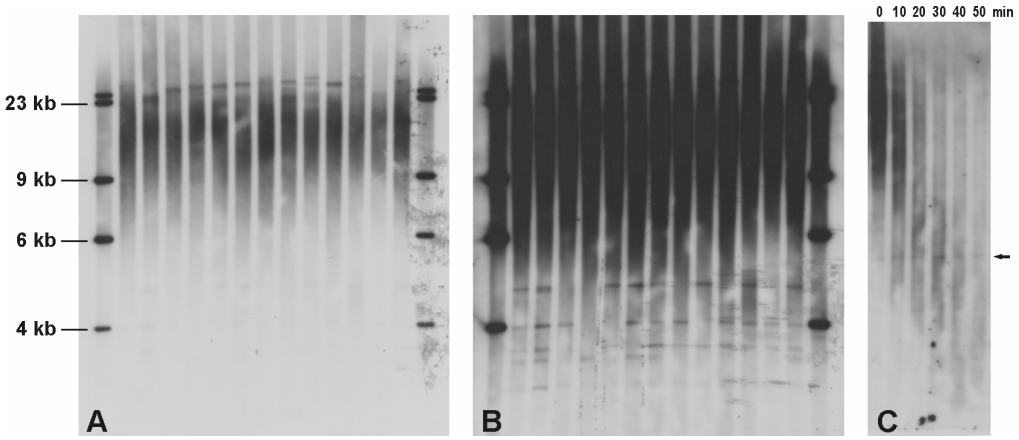

Figure 2.25: Localisation of different telomeric repeats in kakapo. (**A**+**B**) Same blot with different exposure time of the film. Interstitial repeats are only visible at extremely long exposure when telomere signal is completely overexposed. Molecular weight is indicated. (**C**) Digestion with exonuclease *Bal*31. Telomere smear progressively shortens with time of digestion (top), while interstitial bands stay at the same molecular weight (arrow).

A substantial increase of exposure time revealed the present of interstitial repeats in all species examined (e.g. kakapo, Fig. 2.25A+B). To confirm the position of the different fragments within the chromosome, genomic kakapo DNA was digested with *Bal*31 prior to restriction with *Rsa*I and *Hinf*I. *Bal*31 is an exonuclease that progressively digests terminal DNA. As shown in Fig. 2.25C, the telomere smear shifts towards lower molecular weight with increasing digestion time while bands of interstitial repeats do not change. Fig 2.25A+B shows only kakapo as an example, but all other species apart from zebra finch had a similar pattern. In all of them no interference of interstitial and true telomere signal was observed. The interstitial repeats might contain less and/or more degenerated copies of the telomeric sequence and therefore might exhibit less signal intensity as less probes can bind compared to real telomere fragments with mainly true telomeric DNA.

In contrast, zebra finch displayed a unique telomeric signal (Fig. 2.26). All samples showed an intense high molecular band at the same position, indicating that this might be the limit of mobility. This fragments could indeed be ultra long telomeres as described in (Delany et al 2000). In addition, an extensive pattern of interstitial (class I) repeats was found, preventing analysis of class II fragments using denaturing Southern blot.

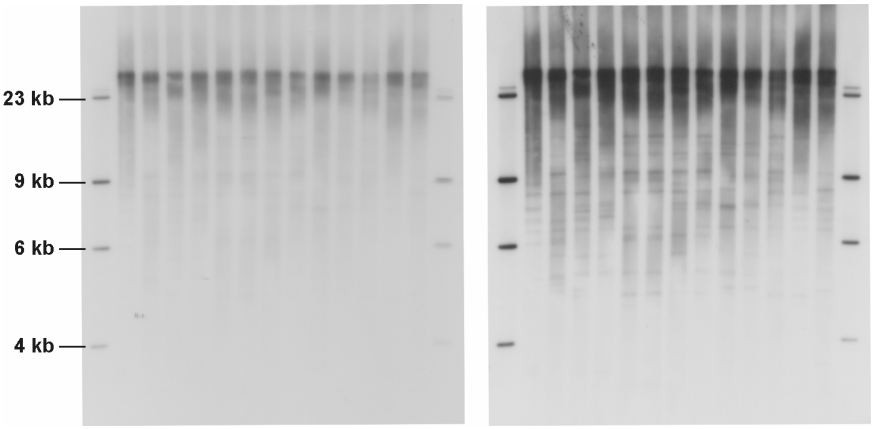

Figure 2.26: Telomeric fragments of zebra finch. A high molecular band at the limit of mobility and interstitial telomeric repeats at longer exposure (**right**) are present. Molecular weight is indicated.

2.4 Discussion

TRF can be a very useful method to measure the telomere length in the field of ecology. The two main drawbacks mentioned in literature are the high quantity and quality of DNA required and the introduction of error due to the subtelomeric part of the restriction fragment (Nakagawa et al 2004; Baird 2005).

2.4.1 DNA quantity required

Required DNA amounts of up to 10 µg (Nakagawa et al 2004) or 10^5 cells (Saldanha et al 2003; Baird 2005) have been reported for TRF. While this might be a limiting factor in clinical studies in humans, it is not when working with blood samples of birds, which have nucleated erythrocytes. The DNA yield from bird blood samples has been estimated to be approximately 350 times higher that from the same amount of mammal blood (Freed & Cann 2006). The 0.5 µg of DNA used for each sample per blot in this study was equivalent to less than 0.5 µl of whole blood using standard DNA extraction methods.

Standard extraction method also yielded high quality DNA in the present study, according to agarose gel and absorption spectrum analysis. Short term storage in lysis buffer did not change the TL estimates compared to liquid nitrogen/-80°C storage.

2.4.2 Subtelomeric part of restriction fragments

There are different estimates of how much the subtelomeric part of the restriction fragments comprises to the estimated TL. Most studies measured a set of sample with TRF and a method supposed to measure only telomeric repeats (FISH or Q-PCR). The intercept of the regression line should then indicate the proportion of the restriction fragment that is not telomeric sequence (Cawthon 2002). Several factors can distort this estimation. First, as shown above, the magnitude of the measured TL is highly dependent of the analysis method. In this case the correlation (R^2) between the methods stays unchanged, but the whole distribution, including the intercept, shifts along the axis of TRF. Second, error rates of the reference methods are often neglected. For example, in Q-FISH all chromosomes of a metaphase spread and several spreads have to be measured to get a mean TL comparable to TRF. Measurements of just a subset of all telomeres in a cell are often used to compare Q-FISH to TRF.

Cawthon (2002) argued that if two samples have the same TL measured by FISH, the difference between the TL of the same samples estimated by TRF should be the influence of different restriction sites of the samples (Cawthon, discussing a figure from Hultdin et al (1998)). As discussed in section 2.1, Q-FISH can have a higher measurement error than TRF, justifying the opposite assumption that the difference in fluorescence for two samples with the same TRF telomere length is due to the measurement error of Q-FISH. In the case of Q-PCR the result might be influenced by interstitial telomeric repeats (ITS) that can not be disting

uished from true telomeres with this technique or single copy gene primers that might amplify more than a single copy locus (see Chapter 5).

Baird et al (2006) estimated approximately 1 kb of DNA between the most distal restriction sides of *Hinf*I/*Rsa*I and *Hph*I/*Mn*lI in human, where the latter ones mark the distal end of imperfect telomeric repeats. This is in the range of the 1.5 kb difference found in the present study between *RasI* and *MseI* in a pooped kakapo reference sample. Although a variability

between individuals cannot be excluded, the 1.5 kb is the mean difference of the 66 pooled birds indicating that a possible variability would be small.

From a biological point of view it is always assumed that only the "real" telomeres are involved in ageing and signalling replicative senescence. But what if imperfect repeats and even subtelomeric regions are also involved? If there are individual differences in restriction site positions they might also be indicative of telomere function as they mark the border between subtelomeric sequences and the repetitive telomere-like sequences at the end of each chromosome.

2.4.3 Interstitial telomeric repeats and ultra long telomeres

Another argument against TRF in birds is the presence of ultra long telomeres (Delany et al 2000). Haussmann & Mauck (2008) used that argument to introduce their TOE method which cuts off much more than only the ultra long class III telomeres (Section 2.2.5.5). Of the six bird species investigated in this study only one (zebra finch) showed TL that was not resolvable by constant field agarose electrophoresis. Not sign of class III telomeric fragments was detected in the other bird species nor in the one fish species included in this study. It is not clear if these class is absent from the studied bird species or the fragments too large to enter gel in constant gel electrophoresis. It has been suggested that class III fragments are retained in the wells under electrophoresis conditions similar to the ones used in the present study (Swanberg & Delany 2003). Either way they did not interfere with TL measurement in most birds.

The same applies for interstitial repeats. Although the telomere probe binds to interstitial repeats in denaturing Southern blot, the intensity of these bands were so low compared to the telomere signal that no bands could be detected with the film exposure time used for TRF analysis. Only interstitial repeats derived from *Mse*I showed higher intensity that might interfere with TRF. *Mse*I cuts a telomeric variants and is therefore likely to produce fragments originating

from the imperfect telomeric repeats region. This assumption is supported by a lower TL estimated with *Mse*I than any other enzyme tested with the pooled kakapo reference sample. The high intensity of interstitial *Mse*I bands could be explained by a higher proportion of telomeric sequence of these fragments compared to "true" interstitial fragments. The use of two frequently cutting and telomeric-unrelated restriction enzymes (*Rsa*I and *Hinf*I) proved to result in sufficient small fragments for TRF analysis without interference of interstitial repeats.

2.4.4 TRF analysis in publications

Several factors ranging from electrophoresis, over restriction through to image quality and analysis influence the outcome of TRF assays. Factors like measurement error, magnitude of TL and relation between TL of different individuals is highly influenced by experimental performance. Although optimization of Southern blots can be painful due to the long time needed to complete one blot (four days with the method used in the present study), it is necessary to pay attention to every step of the protocol to obtain reliable results. This chapter showed that small details can have a huge influence on the estimated TL and telomere distribution.

Unfortunately this is not obvious to all author and reviewers resulting in publications with questionable results due to extensive background (Binz et al 2005; Bonab et al 2006), missing resolution (Schaetzlein et al 2004), overexposure (Ishii et al 2006) or just general bad picture quality (Jennings et al 1999; Lanza et al 2000; Callicott & Womack 2006).

It is especially worrying if bad quality TRF blots are used to emphasise the superiority of a new developed method (Gan et al 2001), or a diagonal stripe of missing signal across the whole blot coincides with a "significant" decrease of TL (Gupta et al 2007). In other publications the presented TL does not correspond to the picture shown (Swanberg & Delany 2003; Jeon et al 2005). Unfortunately more and more authors tend to not publish any picture of their TRF results

and neglect replicates or controls (Appendix Table II.I). If experiments are conducted in this way, TRF can be abused to virtually show whatever the investigator intends to find.

Another common misconception is the overrating of correlations. This applies to the correlation between different methods to measure TL as mentioned before, but also to the correlation between TL and age or population doubling in cell culture. A highly significant correlation between TL of human leucocytes and age (Zhai et al 2006) is meaningless with an coefficient of determination of 0.06 (i.e. 6% of the TL variation can be explained by age). A correlation with the favourite colour of the participants would probably give a higher R^2. Biological conclusions should always be based on the effect size and not on a p-value (Nakagawa & Cuthill 2007).

Also, the effect size of a measured age dependent decline of TL has to be considered in the light of measurement error. For example, Pauliny et al (2006) reported a decline of TL in dunlins (*Calidris alpine*) with age when individuals were remeasured after several years. Of the seven birds presented only two showed a decline of TL that exceeded the mean measurement error. Again, most studies do not even determine the measurement error. As some error sources of the TRF procedure are directional (e.g. unequal weight while blotting, uneven distribution of hybridization or washing buffers, etc.), this can lead to wrong conclusions if the samples are not loaded randomly onto the gel.

The measurement error and the choice of analysis type can greatly influence the magnitude and distribution of estimated telomere length. It is therefore difficult to directly compare results of different publication and a clear description in the methods section should be given. Unfortunately 8 out of 79 papers (10.1%) listed in Appendix Table II.I did not give any information about the analysis method used and another 16.5% are rather vague about the method they used. 51 (64.6%) did not include replicates or other controls in their experimental procedure and 23 (29.1%) did not show a picture of a representative blot. The ones that did measure error rates reported highly divergent values. The highest one comes from (Gan et al

2001) with 17%, but as mentioned before these data are used to compare TRF to an alternative method and the blot shown is of bad quality. Other estimates vary between 1.5% (Grant et al 2001; Haussmann & Vleck 2002; Pipes et al 2006) and 4-8% (Pauliny et al 2006; Kurz et al 2006) for intra or inter gel variation (see Appendix Table II.I). Some authors express the error in terms of coefficient of determination between duplicate measurements with values ranging between R^2=0.35 (Pauliny et al 2006) and R^2>0.9 (Jeanclos et al 2000; Ishii et al 2006; Andrew et al 2006; Fitzpatrick et al 2007). Some of the error rates are hard to believe judging from the pictures provided (e.g. Ishii et al 2006). Interestingly, studies on heredity of TL report a higher measurement error in terms of batch effects of 19% to up to 42% (Slagboom et al 1994; Andrew et al 2006).

All these error rates are not directly comparable because in most cases it is not clear if they depict inter- or intra-gel variation or if they were obtained by one sample replicated or several samples. Also the error depends on the magnitude of the TL measures due to decreasing resolution of agarose gel for higher molecular weight. Nevertheless they give an indication of the exactness of results. Several publications mention measurement in duplicates (Zhang et al 2006), triplicates (Keys et al 2004; Liu et al 2007) or the inclusion of calibration samples (Brouilette et al 2008) without any indication of the obtained measurement error. But the majority does not even present the simplest controls (e.g. one sample repeated on each gel or one sample run multiple times in one gel). Given the variety of error sources described in this chapter it is worrying that conclusions are drawn from these datasets.

That a lower standard deviation does not always mean lower measurement error has been shown in 2.2.5.4. More important is to have a lower proportion of the actual TL range of a study covered by the inter- and intra-gel variation. Using this approach the best analysis method for both datasets used for optimizing TRF in the present study (sea bass, representing low TL and kakapo representing high TL), was the method using a logistic model for gel calibration and the formula $\sum(OD_i \cdot MW_i)/\sum OD_i$. The recommended formula that corrects for multiple binding of

the probe (Ci) showed a higher measurement error in both datasets analysed. The calculated SD/range of 4.98% for European sea bass equals a coefficient of variation of 2.43% calculated from CV of all samples and the intra-gel CV. For kakapo, 9.84% SD/range corresponds to a CV of 4.4% as the mean of intra- and inter-gel variation of one sample ran three times on each gel. The use of the rather uncommon logistic model to calibrate the gel might comprise some comparability to other studies in mainly terms of magnitude of TL, but the example of exponential fit estimated by different programmes shows that one has to be careful comparing absolute values from different publications anyway. The optimized protocol for TRF is described in detail in the Appendix I.III.I and has been used throughout the rest of this study.

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3. Telomere length of kakapo measured by TRF

In this chapter, telomere length of kakapo was measured by telomere restriction fragment (TRF) analysis. Telomere length was not correlated with age, but maternally inherited. Weak correlations between telomere length and fitness parameter, like hatching and fledging success, were obtained, but are most likely due to small sample size and the heavy management of the kakapo population.

3.1 Conservation History

The kakapo (*Strigops habroptilus)* is one of the most enigmatic birds of New Zealand. Once thought to be doomed to extinction, the population is now recovering slowly due to intensive wildlife management. In 2006, the Ornithological Society of New Zealand dedicated a whole issue of their journal Notornis (Volume 53 Part 1, March 2006) to one of the world's rarest birds (Elliott et al 2001).

The kakapo is the only member of the subfamily *Strigopidae* with the closest taxonomic group being *Nestorinae*, comprising the kaka (*Nestor meridionalis*) and the kea (*Nestor notabilis*) (Powlesland et al 2006). The kakapo is a flightless, nocturnal lek breeder and the largest parrot alive. Once abundant across both islands of New Zealand, the species now consists of one managed population currently distributed among two offshore islands around NZ.

The decline of kakapo began approximately 800 years ago when humans settled New Zealand (Clout 2006). Kakapo were not only hunted for food and feathers, but were also the subject of predation by mammal pest species introduced by Polynesian (e.g. kiore *Rattus exulans*) and later European settlers (e.g. ship rat *Rattus rattus*, stoat *Mustela erminea* and domestic cat *Felis catus*) (Powlesland et al 2006; Clout 2006).

In the late 1800's the decline of kakapo was recognized by the then caretaker of Resolution Island, Richard Henry. Between 1894 and 1900 Henry transferred hundreds of

kakapo and kiwi (*Apteryx australis*) from surrounding areas of Fiordland to Resolution Island (Clout 2006). This was the first attempt to use islands as a refuge for endangered birds, which was to become a primary strategy of bird conservation in New Zealand. Unfortunately Richard Henry's quest failed when stoats finally reached Resolution Island in 1990 (Clout 2006).

In the 1970's 18 male kakapo were found near Milford Sound. However, the absent of female birds predicted the end of kakapo. A last chance for the recovery of the species was gained with the discovery of a breeding population of 100-200 kakapo, including females and chicks, on Stewart Island at the southern tip of New Zealand in 1977 (Powlesland et al 1995). This population was carefully monitored, but after 18 birds were killed by feral cats in 1982, the remaining 61 birds were transferred to off-shore islands (e.g. Little Barrier, Maud and Codfish Islands) (Eason et al 2006). However, even on these islands kakapo were not safe, as the kiore on these islands were a constant threat to breeding, killing kakapo chicks and destroying eggs. More intensive management measures were necessary to save the kakapo.

When the New Zealand government realized the threat to the (mainly avian) native wildlife from mammalian predators, eradication programmes were developed to clear regions and islands from these pest species. Kakapo were one of the first beneficiaries (Clout 2006). Today, all kakapo live on offshore islands eradicated of mammalian predators and equipped with thousands of rat traps along their coast lines. All birds carry a radio transmitter and are located on a weekly basis. The management has primarily focused on increasing the population size. Supplemental feeding of females (Elliott et al 2001; Houston et al 2007), 24 hour monitoring of nests, and hand rearing when necessary, has increased the survival rate of chicks (Powlesland et al 2006). As a result, the number of kakapo has almost doubled from 51 in 1996 to 91 in 2008, a great achievement in such a slow growing species as kakapo (Elliott 2006, see below).

However, the number of individuals is not the only factor assuring the survival of endangered species. Increasing evidence suggests that genetic diversity is the most important prerequisite for long term survival of populations (Spielman et al 2004; Jamieson et al 2008).

Conservation genetics has therefore been a integral part of kakapo management for years (Robertson 2006).

3.2 Recovery and management

The kakapo conservation programme started in 1977, when a breeding population was found on Stewart Island. After early failure to protect kakapo by less invasive management, all birds where collected and transferred to predator-free offshore islands and monitored closely (Clout 2006). Although protected from predators, the kakapo population recovered only slowly, mainly hindered by their irregular breeding, every 2-7 years on Codfish Island, the main breeding refuge of these birds (Powlesland et al 2006). In addition, high infertility of eggs (39.7%) and poor hatching success (40.9%) prevented a fast increase of population numbers (Powlesland et al 2006). From all eggs monitored between 1981 and 2005, only 28.3% of chicks actually reached independence, with most of them only surviving through human intervention. Monitoring of nests and supplemental feeding of the mothers increased the survival rate of chicks, but introduced another problem. Robertson et al (2006) found that females receiving unlimited supplemental feeding produced more male than female offspring. Careful evaluation of each female receiving supplemental feeding and adjustment of supplementary food to female body weight led to improved management, achieving both more females in breeding condition and the removal of sex bias in offspring. Recently, it has also been shown that specially formulated food pellets can increase the clutch size in kakapo (Houston et al 2007). Although these measures recruited more female individuals to the breeding pool and possibly increased egg production, they did not lower hatching failure suggested to be a consequence of low genetic diversity in the population (Miller et al 2003; Robertson 2006).

All kakapo but one transferred to offshore islands originated from the Stewart Island population. It is not clear if the population there was natural or introduced by European settlers reported to have freed some individuals in Stewart Island in the 19th century (Powlesland et al

1995). The only bird from the main island, Richard Henry, was found in Fiordland in 1972, and is genetically different from the Stewart Island birds (Robertson 2006). He has produced three offspring, which may provide some genetic rescue (Tallmon et al 2004) for kakapo (Robertson 2006).

Of the 91 kakapo alive, 48 have been born in offshore refuges or under monitoring on Stewart Island and are therefore of known age (Daryl Eason, personal communication). The main goal of the present study was to test if molecular ageing can provide age information for the remaining kakapo of unknown age. Age is currently a key parameter missing for approximately half of the kakapo population (47%), yet this is an essential piece of knowledge for recovery planning.

Age-dependent reproductive senescence has been found in both long and short lived bird species (Moller & De Lope 1999; Catry et al 2006). While many unknown age kakapo are still successfully breeding, poor hatching success could be linked to an ageing population. In fact, Richard Henry, probably the oldest and the only Fiordland kakapo shows signs of reproductive senescence (e.g. less effort put in the construction of track and bowl system and booming, Daryl Eason, personal communication). Comparison of Richard Henry's telomere length (TL) to other birds could help identify individuals that might soon leave the pool of reproductive kakapo and help better predict potential age-related effects on the reproductive performance of the population. Molecular ageing could also shed more light on the age of Richard Henry, who some researchers think might be more than 100 years old (Clout 2006).

Furthermore, some studies suggest a correlation between fitness and telomere length in birds. Haussmann et al (2005) reported higher return rates of tree swallows (*Tachycineta bicolour*) with longer telomeres and Pauliny et al (2006) reported evidence for a correlation between TL and recruitment of offspring in dunlins (*Calidris alpine*). If TL is able to predict similar fitness measures for kakapo this information could be incorporated into the management program and could contribute to a faster recovery of the kakapo population.

3.3 Results

Kakapo blood samples were taken from the tarsus vein and snap frozen in liquid nitrogen. Samples were transferred to the lab and stored at -80° until needed for DNA extraction. Telomere length was measured as described in Appendix I.III.I. One sample was run three times on each gel to provide an internal control for measurement error. The intra- and inter-gel coefficient of variation (CV) was 3.96% and 4.84% respectively. Sixty eight birds have been measured, of which 30 were of known age as they were born on offshore islands or found as chicks or juveniles within the last 25 years. For the remainder, minimum age was estimated using the date they were caught plus 1 to 5 years, based on factors including weight, plumage and first observed breeding attempt (Daryl Eason, personal communication).

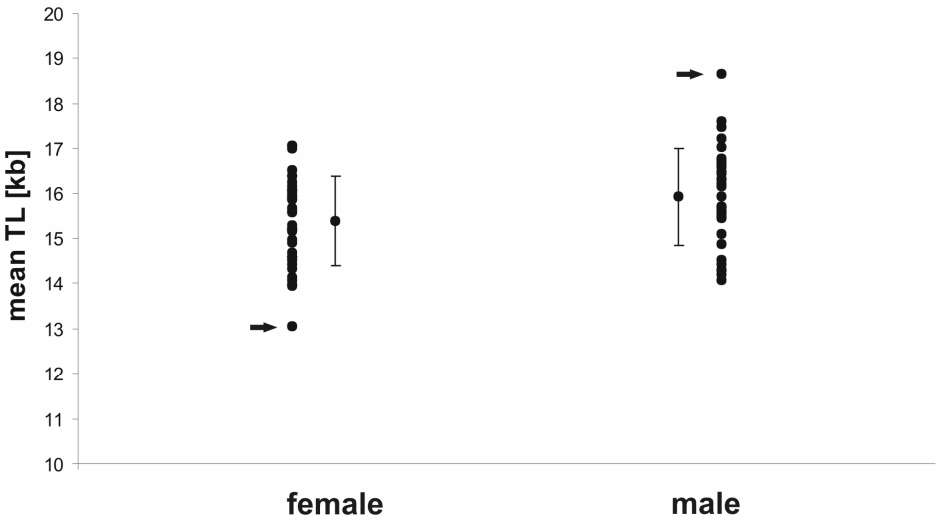

Figure 3.1: Telomere length of male and female kakapo. There was a significant difference between male and female TL for the whole dataset (student's t-test, p=0.031). However, if one of the extreme points from each set (arrows) was removed from analysis, significance vanished. Mean TL and standard deviation are indicated beside the distributions.

The mean TL of all samples was 15.64 ± 1.07 kb (6.82%) and the range was 5.60 kb (13.07 kb to 18.66 kb). There was a significant difference in TL between males and females (Student's t-test p=0.031), but it was mainly based on one female with extraordinary low TL (Jean) and one male with extraordinary high TL (Smoko, Fig. 3.1, arrows). Removal of either of

these individuals eliminated statistical significance. Telomere length was not correlated with age (linear regression $p>0.05$, Fig 3.2).

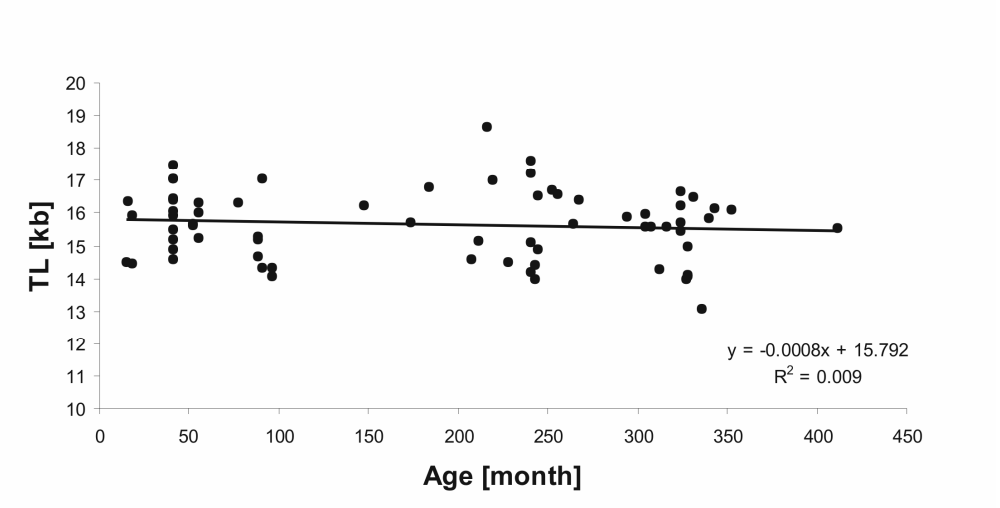

Figure 3.2: Relation between telomere length and age of 68 kakapo.

I also investigated TL change within five young birds that were sampled at two ages: 41 and 55 months (Fig. 3.3). Estimated TL change varied from 70 bp (0.53%) to 410 bp (2.62%) per individual with one individual losing, and four individuals gaining, telomeric sequences. However, all measured changes were within the intra-gel variation of 500bp (3.37%) measured for this specific gel (by running one sample in three different lanes).

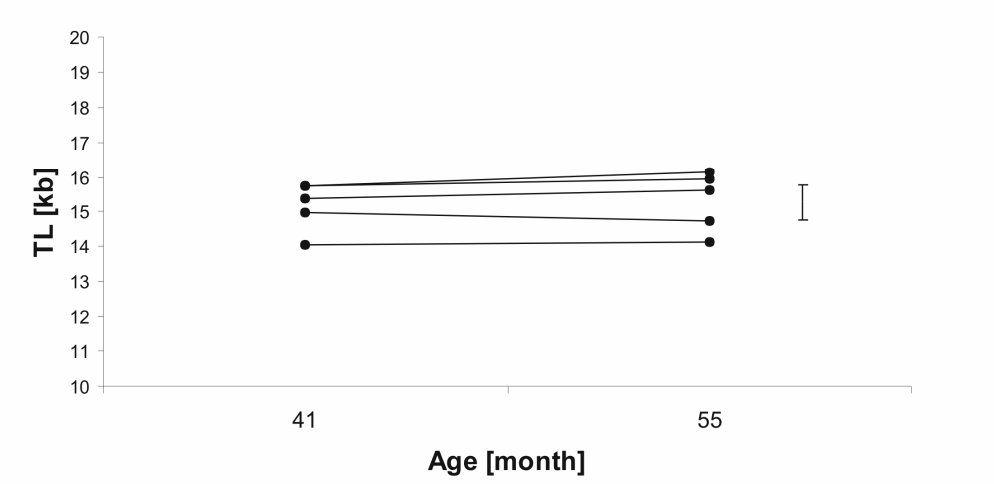

Figure 3.3: Telomere length change within individual kakapo. Telomere length was measured in the same birds at 41 and 55 months of age. All changes were within the intra-gel variation (SD of three replicates, error bar at the right).

The kakapo population has been monitored for over 20 years and various fitness data are available for each bird. The statistical analysis of correlates between telomere length and fitness data, obtained by the national kakapo team, was done by Graeme Elliott (Department of Conservation, NZ). There were 51 fertile eggs with known TL of both parents. Fledging success was negatively correlated with parental telomere length (male χ^2=3.946, DF=1, p=0.047; female χ^2=5.050, DF=1, p=0.025). However, excluding one of the main female breeders (Cyndy, 45 fertile eggs left) reduced the correlation between mother's and offspring's TL to p=0.16 (χ^2=1.97, DF=1). Conversely, a significant relationship emerged between mother's TL and the fertility of eggs when the same female breeder (Cyndy) was excluded (χ^2=2.721, DF=1, p=0.099 (118 eggs) vs. χ^2=5.115, DF=1, p=0.024 (101 eggs)). Telomere length was not correlated with counts of white blood cell or the heterophils/lymphocytes ratio (national kakapo team, unpublished data).

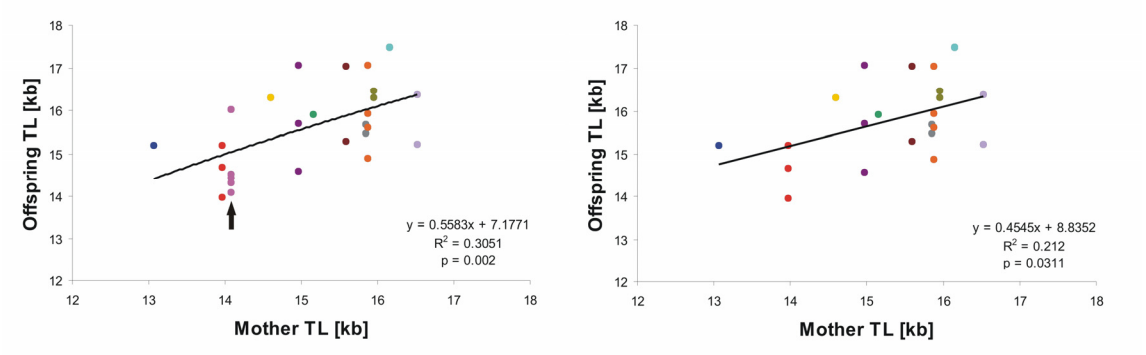

Figure 3.4: Inheritance of telomere length in kakapo. TL of offspring was correlated with maternal TL. Each mother is shown in a different colour. Exclusion of the most influential female (Flossie, arrow) from the analysis did reduce, but not eliminate the significance. P-value obtained by linear regression.

The dataset also allowed analysis of the hereditability of telomere length in 26 pairs where TL of both parents and the offspring was known. Telomere length of offspring was not correlated with parental TL (p = 0.46) but was correlated with maternal TL (p = 0.003, general linear model (GLM), DF = 25). Fathers were excluded from further analysis and three additional pairs where only maternal and offspring TL was known were included. Fig. 3.4 shows a linear regression of offspring and maternal TL. Due to small sample size, multiple offspring of one

mother was included, but the exclusion of the most influential and productive female (Flossie, Fig. 3.4 arrow) did not eliminate the significance of the linear regression (linear regression, p=0.002 and p=0.0311).

A full pedigree analysis would be nessesary to calculate the heritability (h^2) of TL. Unfortunately the pedigree of the Kakapo population, although under construction, is currently not available. Alternatively, h^2 can be estimated using the slope of the regression between mother TL and mean TL of all her offspring. Using this method, maternal inheritance of telomere length h^2 is 0.5 in kakapo.

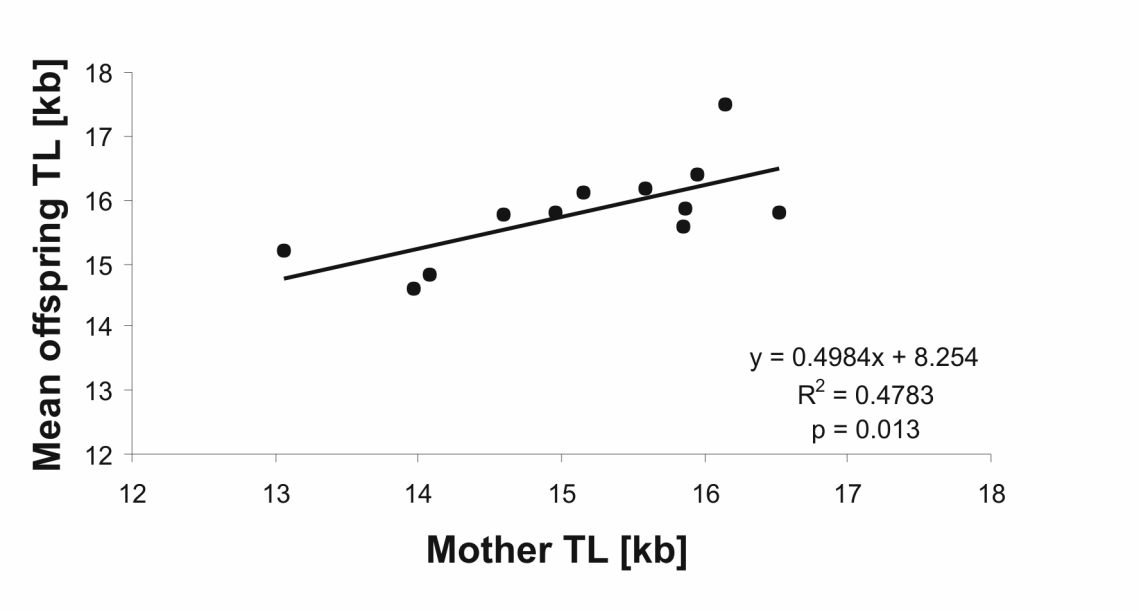

Figure 3.5: Heritability of telomere length between mother and chicks. TL of mother was plotted against the mean TL of all her offspring. The heritability (h^2) equals the slope of the regression.

3.4 Discussion

In the last 20 years molecular biology has become an important tool in ecology and wildlife management. Phylogenetics and phylogeography help identify different species and populations within their natural habitat (Avise 2004), molecular sexing and paternity tests are routinely used in field studies and conservation (Robertson & Gemmell 2006) and the

assessment of genetic diversity is included in an increasing number of wildlife management plans (Jamieson et al 2008).

Kakapo conservation is a good example of incorporation of molecular biology in species recovery planning, and the importance of genetics to conservation (Robertson 2006). Molecular sexing approaches have identified a sex bias driven by supplementary feeding, and assisted in removing this bias without losing the benefits of the feeding (Robertson et al 2006). Molecular paternity analysis of surviving and dead chicks has and will help identify individual bird's reproductive success, with a view to managing breeding attempts to maintain existing genetic variation in the population (Robertson 2006).

Telomere length of kakapo appeared to differ between sexes, but the correlation disappeared if just one of the extreme data points for either females or males was excluded (Fig. 3.1). Indeed, overlapping standard deviations suggest that if there is an effect of sex on telomere length, it is minimal. Sex specific differences could arise from differences in diet and metabolic rate, inducing varying levels of oxidative stress (Holmes & Ottinger 2003). However, body mass specific metabolic rates have been found to be equal in adult male and female kakapo (Bryant 2006). Alternatively, hormonal differences between male and female birds could influence telomere length, as suggested in humans (reviewed in Demerath et al 2004), but little is known about the endocrine system of kakapo. As a difference between male and female TL has not been reported in any bird species (e.g. Haussmann & Vleck 2002; Hall et al 2004), the observed pattern in kakapo probably emerged by chance.

Unfortunately, there was no correlation between telomere length and age in kakapo. Telomere dynamics in birds younger than 15 months remains unresolved as no samples were available for that range, due to the species' endangered status, irregular breeding and management practices. Higher telomere rate of change (TROC) has been reported for young birds compared to adults (Hall et al 2004; Pauliny et al 2006; Juola et al 2006), and might therefore enable molecular ageing of younger birds, but this is not useful in kakapo as juveniles

are easily distinguishable from adults by their plumage up to an age of 14-16 months (Daryl Eason, personal communication).

Age-independent telomere length (TL corrected for age) has been linked to reproductive success and survival in birds (Haussmann et al 2005; Pauliny et al 2006). As kakapo did not show any sign of TL decline or increase with age (Fig 3.2), a correction was not necessary and TL could be directly tested for effects on fitness parameters.

Survival among adult kakapo is remarkably high. The mean annual survival rate of adult birds transferred to offshore islands between 1982 and 2005 was 0.99 (Elliott 2006). Likewise, the annual survival rate of juveniles in the present managed population was estimated at 0.91 (Elliott 2006). In comparison, the annual survival rate of wild birds is typically between 0.5 and 0.8 (reviewed in Sandercock et al 2000). A correlation between TL and survival rate in kakapo could therefore not be tested.

Other fitness data available included weight, reproductive success and blood cell counts, but most of these data are prone to distortion by human interference through wildlife management. Weight is adjusted by supplemental feeding to ensure minimum required breeding condition and might be dependent on the island housing the particular bird. Which of the currently two islands an individual inhabits together with vaccination measures carried out regularly, might also influence blood cell counts and other haematological component analyses. No relationship was found between TL and white blood cell count, heterophils/lymphocytes ratio or estimated breeding season weight (data not shown).

Correlations between reproductive success and telomere length appeared to be marginal and heavily influenced by data points from single birds. The higher fledging success of chicks from parents with lower telomere length could be explained by better nutritional condition of the parents. Demerath et al (2004) suggested that oxidative stress and accelerated cell turnover induced by rich diet can result in age-independent shortening of telomeres. In this case, parents in good nutritional conditions might have higher reproductive success, but lower telomere

length. In kakapo, like other intensely managed threatened species, fledging success is dependent on human assistance such as egg incubation if the mother leaves the nest for too long, supplemental feeding of chicks and hand rearing or medical care for chicks. Consequently, the relationship between parental TL and fledging success is highly variable as it can be eliminated or even caused by human interference (Elliott et al 2001; Robertson et al 2006).

Another factor that can bias fitness data is the low heterozygosity of kakapo (Robertson et al 2000; Miller et al 2003). Low hatching and fledging success has been associated with low genetic diversity in birds (Spottiswoode & Moller 2004). Detailed information of relatedness between all kakapo is currently being obtained by comprehensive microsatellite analysis and will be available for comparison with telomere data soon (B. C. Robertson, personal communication).

Maternal telomere length was highly correlated with offspring TL: approximately 30% of variation in offspring TL could be explained by maternal TL. Despite small sample sizes and skewed production of chicks to a few females (i.e. up to 7 chicks were from one mother), the relationship between maternal and offspring TL was maintained even when the most influential (7 chicks) female was removed from the analysis (Fig. 3.4).

Inheritance of telomere length has only been investigated in the last few years and mostly in humans. Although all studies found heredity of TL with estimates of 36-90% (reviewed in Baird 2006; Baird 2008), there is substantial confusion in the field about the mode of inheritance and quantitative trait loci associated with TL. While Nawrot et al (2004) reported maternal inheritance of TL, Njajou et al (2007) and Nordfjall et al (2005) suggested paternal inheritance. Further more, linkage analysis by Vasa-Nicotera et al (2005) identified different loci than Andrew et al (2006) in their study. Even mutations within identified quantitative trait loci, the promoter region of the catalytic subunit of telomerase (Matsubara et al 2006a), were found to have some effect in one population (Matsubara et al 2006b), but not in another (Nordfjall et al 2007). While most of these discrepancies can probably be explained by different sample size and

choice (Baird 2008), they bring into question the existence of an universal genetic mechanism controlling heritability of telomere length.

In fact, a comprehensive study identified more than 100 genes associated with telomere length in yeast (Askree et al 2004). In mice, a minimum of five loci have been suggested to contribute to telomere length regulation (Manning et al 2002). Loci suggested to influence TL include regulatory regions for telomerase (Matsubara et al 2006a), genes involved in oxidative stress defence (Nawrot et al 2004) and genes involved in the endocrine system (Aviv 2002).

The present study of kakapo is the first to report inheritance of telomere length in a bird species. The mechanism or mode of heredity is still to be determined. The exclusive linkage between mother and offspring seems to resemble findings of paternal linkage in humans (Nordfjall et al 2005; Njajou et al 2007) as females are the heterogametic sex (ZW) in birds. Alternative explanations might include gene imprinting (Nordfjall et al 2005), a roll of maternally inherited mitochondrial genes (Nawrot et al 2004) or maternally regulated telomerase activity in the egg-cell and zygote.

To clarify the situation, an investigation of a larger non-endangered bird population is necessary. The exclusion of multiple offspring of one male or female would provide more statistical security and might further resolve the pattern of TL inheritance in birds.

In conclusion, telomere biology could not directly aid kakapo conservation. One reason for that might be the blurring of relationships between TL and fitness measures due to intense management of the population. While the failure to age kakapo is unfortunate, lacking correlation between TL and age might be seen as positive, as it means that kakapo might not be under the restraint of telomere length induced replicative senescence and the resulting onset of age related diseases. There might be other mechanisms of inducing senescence, as probably seen in Richard Henry, but these might be less severe than the effects proposed for TL-induced senescence (Wright & Shay 2005). The low mortality rate of adult kakapo (Elliott 2006) seems

to support this theory. If and how birds with constant telomere length avoid cell immortalization and tumour development, as suggested by Hall et al (2004), remains to be discovered.

At this stage, telomere length is not a measure that can help kakapo recovery. Telomere length might be independent of reproductive success or the effect might be masked by factors like low genetic diversity. Investigation of natural, non-managed bird species (preferably parrots) might provide a clearer picture and result in the incorporation of telomere biology in kakapo in the future. Until then, priority should be given to ascertaining the main factors responsible for low breeding success. For example, it is important to assess the role of genetic variation and genetic similarity of breeding pairs in poor hatching success (Robertson 2006). A comprehensive study using 30 microsatellite markers is currently being conducted (B. C. Robertson, personal communication) and might also help to clarify the effects of inbreeding on telomere length.

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4. Utility of telomere length in ecology

4.1 Telomere length and age

This Chapter assesses the utility of molecular ageing in birds by comparing the results obtained from kakapo (Chapter 3) with several other New Zealand bird species. The use of TL for ageing and prediction of reproductive success or survival rate is discussed using results from the present study and published studies on other bird species.

4.1.1 Telomere length of additional New Zealand bird species

In kakapo, telomere length (TL) did not change with age (Chapter 3). To determine if this is a characteristic of parrots (as parrot TL has not been examined before), this study was expanded to one of the two closest relatives of kakapo: the kea (*Nestor notabilis*). Like kakapo, kea are also extremely long lived (the oldest bird in this study was 27 years old). However, their ability to fly and their omnivorous diet results in a higher metabolic rate compared to kakapo (Bryant 2006). Consequently, kea should be expected to show a higher level of oxidative stress then kakapo that could induce or accelerate the loss of telomeric repeats. Twenty samples from kea ranging in age from 0.5 to 27 years were measured using the optimized TRF protocol outlined in Appendix I.III.I. Intra- and inter-gel variation was 3.8% and 2.8% (based on one sample run on both outer lanes of each gel), which was comparable to the kakapo assay (3.96% and 4.84%, Section 3.3). Kea showed a large range of TL (3.72 kb or 41% of the maximum), but there was no correlation between TL and age for either the full sample set or for the subset of birds younger than 5 years (Fig. 4.1).

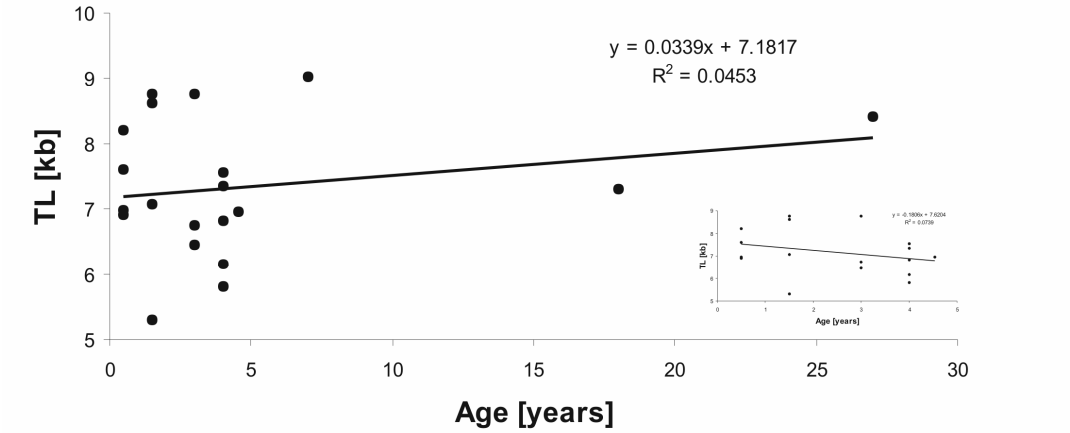

Figure 4.1: Telomere length of kea. A significant correlation by linear regression between TL and age was absent for both the total data and a subset consisting of only juvenile birds (inlay).

Molecular ageing is only useful if morphological differences (e.g. plumage, size, weight) that could indicate the age are absent. In birds, this is usually the case after the juvenile stage (Higgins et al 2006a, see below). Nevertheless, to test if ageing based on telomere length works at all, it is useful to include chicks in an initial measurement. One blot with the youngest and the oldest birds available can show if and how much change in TL occurs in a species. The variation within and between the age classes can then be used to decide if further investigation of the species is worthwhile.

Therefore, three additional New Zealand bird species were examined using chicks and adult birds. The Buller's albatross (*Thalassarche bulleri*) is another long lived species, like kakapo and kea, with some individuals known to reach more than 20 years of age (Fig. 4.2). TL of five chicks and five adult birds were measured on one blot (Fig. 4.2). TL ranged from 8.31 kb to 19.42 kb (range 57.21%) and was significantly correlated with age (linear regression, $p<0.01$). The maximum variation within the chick age class (34.58%) was similar to the difference between the means of both classes (35.9%).

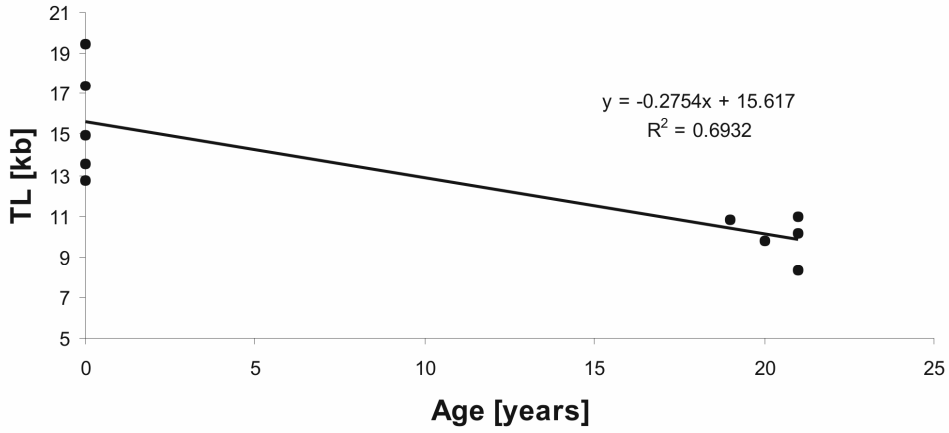

Figure 4.2: Telomere length of Buller's albatross. TL was significantly correlated with age (linear regression, $p<0.01$).

The saddleback (*Philesturnus carunculatus*) and New Zealand robin (*Petroica australis*) are two endemic New Zealand passerine species with short life spans (3-6 years) compared to kakapo, kea or Buller's albatross. Samples from old birds were limited resulting in a broader age class of adults. Linear regression showed a significant decrease of TL with age for both species ($p<0.05$, Fig. 4.3). Telomere length ranged from 6.96 kb to 7.91 kb (11.97%) for saddleback and from 11.05 kb to 17.78 kb (37.85%) for NZ robin.

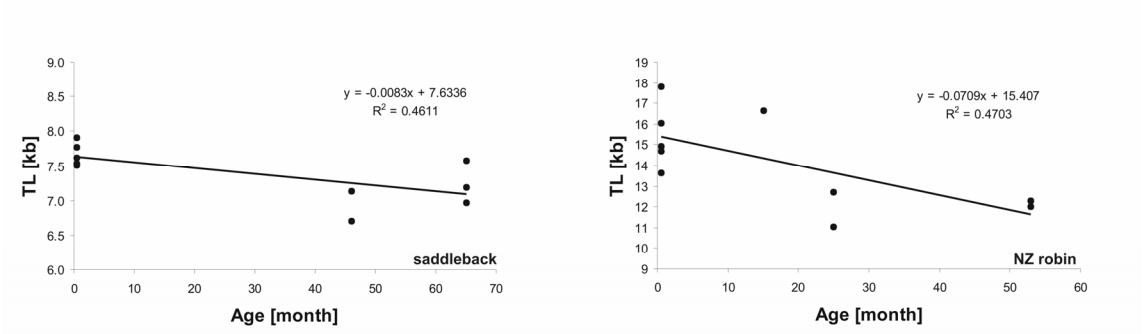

Figure 4.3: Telomere length of saddleback and NZ robin. Linear regression showed a significant decrease of TL with age in both species ($p<0.05$).

Buller's albatross, saddleback and NZ robin showed a decline of TL with age in the sample set analysed. To assess the usefulness of molecular ageing in these species we can use a

linear regression line if we assume that the telomere rate of change (TROC) is constant throughout the lifetime of the birds. Although this assumption is probably not true, it is the 'best case scenario' for molecular ageing. As discussed in Section 1.2, the TROC has been found to be higher early in life in most species. Hall et al (2004), Pauliny et al (2006) and Juola et al (2006) reported a decline of TL between chicks and adults, but no or little change of telomere length within adults, limiting the ability of molecular ageing to estimate age of adult birds. If molecular ageing under the assumption of a constant TROC is found to be not applicable in a species, then it is even less likely to produce reliable results in the more realistic case of TROC decreasing with age.

For Buller's albatross, age of an individual accounted for 69% of the observed variations in TL (Fig. 4.2). However, the range of TL in chicks was very large indicating that estimation of individual age might be difficult due to high TL variations between age matched individuals (Fig. 4.4). Age explained almost half of the variation in TL found in saddleback and NZ robin (Fig. 4.3), but the ranges of TL of chicks and adults overlapped each other (Fig. 4.4 and Table 4.1).

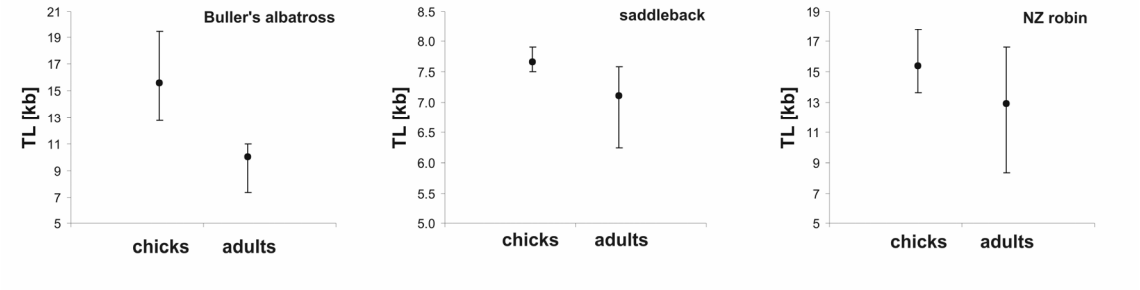

Figure 4.4: Mean telomere length and ranges for Buller's albatross, saddleback and NZ robin. Bars show the range of TL for each age class.

The data analysis method used in this study was chosen based on lowest measurement error and highest coefficient of determination (see section 2.2.5.4). It is important to note that the latter criterion might lead to an overestimation of the coefficient of determination (R^2) and

therefore a to a too optimistic estimate of the utility of molecular ageing in a species. Also, an intra-gel variation of 2.11 kb (12.77%) for Buller's albatross, 0.26 kb (3.77%) for saddleback and 1.42 kb (9.3%) for NZ robin compromise the precision with which age could be determined from telomere length.

Table 4.1: Telomere length and coefficient of determination for additional NZ birds. Five chicks and five adult birds were measured on one blot for each species.

Species	R^2	age class	mean TL [kb]	min TL [kb]	max TL [kb]
Buller's albatross	0.69	chicks	15.61 ± 2.78	12.71	19.42
		adults	10.00 ± 1.07	8.31	10.97
saddleback	0.46	chicks	7.67 ± 0.17	7.50	7.91
		adults	7.12 ± 0.32	6.71	7.58
NZ robin	0.47	chicks	15.42 ± 1.57	13.64	17.78
		adults	12.94 ± 2.15	11.05	16.63

4.1.2 Assessment of molecular ageing

The age of a study object is one of the key parameters in ecology and wildlife management, yet, it is probably the most difficult one to obtain without destructive sampling or many seasons of study. Molecular ageing was promised to be an easy and minimally invasive method to age birds and possible other taxa (Haussmann & Vleck 2002). Although an increasing amount of evidence has been found that age estimation using telomere length lacks the necessary precision (Juola et al 2006) or even its prerequisite (change of TL with age) (Hall et al 2004) to be useful in birds, it is still promoted by some authors as a widely applicable and robust tool (Haussmann & Mauck 2008a).

The present study presents data on telomere length in five different New Zealand bird species (Table 4.2) using different age ranges depending on the goal of the assay and availability of samples. Most extant individuals of the kakapo population were available for this study. However, due to the species' endangered status, irregular breeding and management practices,

no blood samples of kakapo less than 15 months old were available. Consequently, it is unclear whether TL decreases early in life as reported in other birds that show constant TL within adults (Hall et al 2004). In the case of kakapo, molecular ageing of young birds is not a priority because chicks and juveniles are readily distinguishable from adults by their plumage (Powlesland et al 2006; Daryl Eason, personal communication). The main focus of molecular ageing in kakapo was age estimation of birds found as adults more than 20 years ago (i.e. birds of unknown age), however, this could not be achieved.

Table 4.2: Average telomere length, range, R^2 and TROC of bird species in this study.

Species	**average TL [kb]**	**TL range**		**R^2**	**TROC [bp/year]**
		[kb]	[%]†		
Buller's albatross	12.80	11.11	57.21	0.69	-275*
kakapo	15.64	5.95	29.98	0.01	-9.6
kea	7.34	3.72	41.20	0.05	+34
NZ robin	14.18	6.73	37.85	0.47	-850*
saddleback	7.39	1.2	15.17	0.46	-99.6*

† calculated as % of the maximum TL, * change significant by linear regression

A maximum of telomere rate of change (TROC) is found early in life in many birds (Hall et al 2004; Pauliny et al 2006; Juola et al 2006) and other species (Zeichner et al 1999; Brummendorf et al 2002; Baerlocher et al 2007). Therefore, very young individuals were included in the analysis of TL for an initial test of molecular ageing in several bird species, on the assumption that the difference between the oldest and the youngest birds should provide an estimation of the maximum possible usefulness of molecular ageing in a species.

The youngest birds available from kea were half a year old (chicks were sampled as fledglings during winter) and only a few birds older than five years were available for analysis. Nevertheless, neither the whole sample set nor the birds younger than five years showed a decline of telomere length. It is unknown if telomeres are shortened in the first six month of life, but again, this period is not relevant to molecular ageing, as juveniles are distinguishable from adults by their plumage until an age of approximately 1 year (Higgins 1999). The lack of

correlation between telomere length and age in kakapo and kea might be inherent to NZ parrots or parrots in general. Presently no telomere data are available from any other parrot species.

To further test the possibilities of molecular ageing in birds, TL of Buller's albatross, another long lived species, was measured. Using the youngest (chicks) and oldest birds (adults) available, a strong relationship was found between TL and age (Table 4.2). Assuming a linear decline with age (best case scenario, see section 4.1.1), this difference might be sufficient to estimate the age of individual birds based on their TL. Unfortunately, this assumption could not be tested in this study, as no intermediate samples were available. In wandering albatross, a decline of TL has been reported between chicks and adults, but not within adult birds alone (9-42 years, Hall et al 2004). Wandering albatross chicks had a high variation of initial TL similar to that observed Buller's albatross. However, unlike the present study (Fig. 4.2 and 4.4), adult wandering albatross also displayed high variation in TL (Hall et al 2004).

A decreased variation of adult telomere length compared to chick TL has been reported in Leach's storm-petrels (*Oceanodroma leucorhoa*) (Haussmann & Mauck 2008b). The authors suggested that the narrowing of TL distribution in older birds is caused by selection against birds with short telomeres, which results in a truncated distribution for adults because birds with lower TL are eliminated. However, the selection against short telomeres was suggested to result in the observed increase of TL with age, when measured by cross sectional sampling (Haussmann & Vleck 2002). In contrast to this observation, TL of Buller's Albatross declined with age. Nevertheless, assuming a general shortening of telomeres with time, a selection factor eliminating birds with short telomeres, for their age, could explain a narrowing of the distribution. My sample size is too small to draw any conclusions, other than that Buller's albatross might be a good species to examine further telomere length dynamics.

For molecular ageing, the roll of age in TL can be assessed by considering the coefficient of determination between TL and age. The value of 0.69 for Buller's albatross was high compared to other birds in both the present study and previously published studies (Table 4.3).

Only the frigatebird (*Fregata minor*) has shown a higher R^2 (0.82) when using square-root transformed age (Juola et al 2006). The frigatebird study was the only one to apply molecular ageing to birds of unknown age. The authors constructed a calibration curve based on 36 known birds to age 81 unknown birds. However, age classes for a given TL based on a 95% confidence interval were too broad to produce any useful information on age and values derived directly from the standard curve yielded age estimations of up to 120 years for a species with a maximum confirmed life span of 44 years. The authors concluded that molecular ageing was not feasible in this species due to change of TROC throughout life and high intra-age variation of TL, despite age explaining 82% of variation in TL (the highest R^2 ever published for birds).

Table 4.3: Coefficient of determination and TROC in different studies.

Species	**R^2**	**n**	**TROC [bp/year]**	**Reference**
zebra finch	0.45	27	-516[a]	(Haussmann & Vleck 2002)
tree swallow	0.34	N/A ≈ 45	-391[a]	(Haussmann et al 2003)
Adelie penguin	0.55	N/A ≈ 25	-235[a]	(Haussmann et al 2003)
common tern	0.61	N/A ≈ 40	-57[a]	(Haussmann et al 2003)
Leach's storm-petrol	0.66	N/A ≈ 30	+75	(Haussmann et al 2003)
European shag	0.35[b]	144	-300	(Hall et al 2004)
Wandering albatross	0.36[b]	92	-160	(Hall et al 2004)
sand martin	0.55	20	power fit function	(Pauliny et al 2006)
dunlin	0.17	28	power fit function	(Pauliny et al 2006)
frigatebird	0.82	36	square-root transformed age	(Juola et al 2006)
Buller's albatross	0.69	10	-275	present study
kakapo	0.01 [b]	68	-9.6	present study
kea	0.05 [b]	20	+34	present study
NZ robin	0.47	10	-850	present study
saddleback	0.46	10	-99.6	present study

[a] unclear how TL was measured, [b] no correlation found amongst adults,

The two passerine species examined in the present study showed coefficients of determination around 0.5. As with Buller's albatross, chicks were compared to the oldest birds to obtain the maximal change of TL with none or few intermediate samples. In both species, the range of TL for chicks and adults overlapped each other (Fig 4.4). The TL range of adult NZ robins even included the mean TL of chicks. Although this was mainly due to one intermediate sample with long telomeres (Fig. 4.3), it highlights the high age-independent variation of TL.

Cleary, even under the assumption that TL shortening is linear with age, a precise (or even approximate) age determination from the regression obtained is not possible in these species.

Based on the data presented for the five species examined in this thesis, a rapid decline of TL early in life cannot be ruled out. Several published studies suggest that such a pattern exists in many birds (Hall et al 2004; Pauliny et al 2006; Juola et al 2006). A higher TROC in young birds would favour age estimation in young birds and perhaps hinder estimation in adult birds. Unfortunately, the greatest need for molecular ageing is for adult birds, as young ones are often distinguishable by plumage (until approximately one year of age in saddleback (Higgins et al 2006b) and NZ robin (Higgins & Peter 2002).

It would have been nice to have samples covering the whole life span of the birds examined (i.e. additional chicks for kakapo and kea and intermediate aged birds for Buller's albatross, saddleback and the NZ robin), but these additional data points were not essential for the purpose of this study: assessment of the utility of molecular ageing. The question of whether TROC changes throughout the lifetime of birds is better addressed in model populations with less limited sample availability and stronger control of environmental variables.

The current study showed that no significant change of telomere length occurred in adult kakapo and kea and that while TL declined in saddleback and NZ robin, TL change was not feasible for molecular ageing in the species examined. Although the sample size used was very limited in some species, the large age span used to calculate maximum change of TL should have indicated if enough difference exists to age individual birds. It remains unclear if TL might be informative in molecular ageing of Buller's albatross, but comparison with studies of other long-lived birds (e.g. frigatebird) suggest that estimation of age would not be reliable in this species.

Two major drawbacks hamper molecular ageing. First, methodological problems inherent to Southern blot and other methods compromise the detection of small differences of TL between birds (see Chapter 2 and 5). At a population study level these measurement errors might not be strong enough to mask patterns of telomere dynamics, so that trends are detectable within

whole sample sets, but on the individual sample level, such errors prevent precise estimation of age. New improved methods to measure TL are needed to address this issue.

Secondly, significant intra-age differences in TL have been found in all species examined for molecular ageing. Some of these differences have been linked to survival rate and fitness parameters of individual birds (Haussmann et al 2005; Pauliny et al 2006; Haussmann & Mauck 2008b). Although age and fitness have been suggested as two parameters that can be analysed simultaneously using telomere length (Haussmann & Mauck 2008a), they are widely exclusive. Age determination based on TL can not be precise if three life history variables (TL as chick, mass/skeletal size, seasonal effects) together explain 61% of variation in telomere length change (Hall et al 2004). Although this number refers to change of TL and not TL itself, it still demonstrates that life history traits may alter TL of individual birds substantially and independently of age. The identification of maternal heritability of TL in kakapo adds another variable that can influence an individuals telomere length.

The issue of methodological problems has recently been addressed by a publication suggesting to measure only a subset of telomeres (the shortest ones) (Haussmann & Mauck 2008a). This method assumes a decline of telomere length with age and searches for the part of the telomere distribution that shows the best correlation with age (Haussmann & Mauck 2008a). Apart from the various methodological problems that arise from this method (discussed in section 2.2.5.5), it is not clear if the assumption of TL decline with age is true in birds. A variety of factors other than age have been found to influence TL in birds (e.g. inheritance, oxidative stress, telomerase activity, see section 4.3). We should be careful not to measure something that might not be there.

In conclusion, molecular ageing based on telomere length is not reliable for ageing birds in its current form. The methodological problem of high error rate might be avoidable by careful optimization and development of new methods, but intra-age variation and the influence of environmental and genetic factors on telomere length compromise precise prediction of age

based on telomere length. However, TL might be useful as a marker for past and present stress factors experienced and the genetic predisposition of individuals to deal with such stressors. (see below).

4.2 Telomere length and fitness

Telomere length has been linked to both, survival rate (Haussmann et al 2005; Pauliny et al 2006) and reproductive success (Pauliny et al 2006) in birds. In tree swallows (*Tachycineta bicolour*), birds in the quartile with longest telomeres had a five times higher probability to return to their breeding ground each year than the birds of the quartile with shortest telomeres (Haussmann et al 2005). This result should be interpreted with caution as the study used both, small sample size and combined data from different TRF protocols (and it is not clear what exactly the difference was). Pauliny et al (2006) used the date when birds were last seen as minimum life span to correlate with residual (age adjusted) TL in sand martins (*Riparia riparia*). Given an average difference of 4.4% between replicates of a subset of the samples tested (Pauliny et al 2006), all but one of the residual TL used for analysis of survival lay within the measurement error. Likewise, for the correlation between tarsus length and reproductive success with TL in dunlins (*Calidris alpine*), all but one of the data points are within the measurement error range (Pauliny et al 2006). In addition, the correlation between reproductive success and TL in male dunlins was only significant if reproductive success was defined as having at least one offspring opposed to the real number of offspring. The pattern observed might still be real, but replicates and preferably triplicate measurements of the samples should be done, if the estimated effect is based on differences of TL within the measurement error.

Telomere length might well be a marker for fitness in birds. If reproductive success can be predicted based on individual TL, this could be extremely helpful for the design of breeding programmes to rapidly increase the number of individuals of endangered species. A combination

of TL data with analysis of heterozygosity could lead to a species management plan that aims to address two of the main aspects of endangered species survival: increasing the number of individuals and preserving the genetic diversity of the population (Jamieson et al 2008). In this case, the presented findings of heredity in kakapo, if confirmed in other bird species, would be useful to further refine management and accelerate species recovery. The field of TL as a measure of fitness is new and we need more data on more species to draw general conclusions, but the potential benefits are large enough to justify further investigation of a relationship of telomere length and fitness in birds and other species.

4.3 Discussion

It is unclear if telomere length changes over time in birds. The present findings and those of others (Hall et al 2004) suggest that TL of adult birds does not decline with age in a cross-sectional setup. TL change might be absent or masked by high variation between age matched individuals. Longitudinal studies of individual birds are necessary to investigate if TL changes over time within adult birds. There was no change in TL in five kakapo measured at 41 and again at 55 month of age. Pauliny et al (2006) reported a decrease of TL in individual dunlins between 6 and 12 years, but most differences found were within the measurement error of the assay. Other studies found a low, but significant, change of TL in adult birds in cross-sectional analysis (Juola et al 2006), but some of them are methodologically open to criticism (Haussmann & Vleck 2002; Haussmann et al 2003; Haussmann & Mauck 2008b, see 2.2.5.5 and 4.1.2).

If TL is constant in adult birds, then environmental factors, and genes modulating their impact on TL (e.g. response to oxidative stress), can only act on TL in chicks. The early period of life, together with TL of the mother might therefore be the most important determinants of TL in birds. If reports about correlation between TL and reproductive success (Pauliny et al 2006) and survival rate (Haussmann et al 2005) find confirmation in further studies, we might have to give chick-stage of development more importance in ecological studies. Environmental factors

changing TROC in chicks would have a great influence on the fitness of the bird throughout its whole lifespan.

Molecular ageing was heralded as a major tool in field studies of animal populations, but it might not be feasible at all. Correlations of TL with lifespan (Haussmann et al 2005) and reproductive success (Pauliny et al 2006) are typically based on low sample sizes, slightly to non-significant p values, and calculated from TL differences within the measurement error (Pauliny et al 2006). Also, new methodological improvements have been designed based on the assumption of TL decline (Haussmann & Mauck 2008a) which might not be true (Hall et al 2004; Pauliny et al 2006, present study). There may well be some merit to molecular ageing, but studies need to refocus on the basic questions, not the least being validation of telomere change with age.

The first step in the process would be to ensure a high reproducibility of TL measurements. All available methods are prone to measurement errors, which lie in the range of the effect size in many species (see section 2.1). An improvement of existing methods or new methods to measure TL with lower measurement error are needed. Then we can test the basic assumption of telomere loss with age, presumably by longitudinal studies in different bird species. Based on this and the extent of TL variation within age matched individuals, we can decide if molecular ageing is a useful tool in ecology. There is also a need for more data to confirm or reject a correlation of TL and survival rate or reproductive success. A survival study announced by Haussmann et al (2005) monitoring 100 birds could bring clarification, but is not available yet.

Lastly, new protocols might help overcome the shortcomings of current telomere analysis. Due to the lack of a nucleus in mammalian erythrocytes, they can not be utilized for telomere length analysis in mammals. Most investigations based on mammal blood samples therefore measure peripheral blood mononuclear cell (PBMC) telomere length (see Appendix Table II.I). Correlations of TL and mortality or disease susceptibility have been suggested to be

mediated by the activity of the immune system (Goronzy et al 2006). In birds, telomere length of white blood cells might also be more informative in terms of age and stress response and show a more pronounced pattern. It might be that the information is all there, we are just measuring the wrong cells.

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5. Telomere length measured by quantitative real-time PCR

This chapter reports telomere length of kakapo measured by Q-PCR. The data obtained by Q-PCR did not correlate with data obtained by TRF. PCR amplification efficiency is identified as the main caused of unreliable results of Q-PCR in kakapo and further discussed.

5.1 Introduction

Telomeres were thought of being impossible to amplify by PCR due to their repetitive nature until Cawthon presented a cleverly designed primer set in 2002. He introduced several mismatches that prevent the two primers from annealing to each other, but still exhibit enough affinity to the telomeric sequence to facilitate PCR amplification. The amount of amplified DNA measured by telomere real-time PCR (or quantitative PCR (Q-PCR)) is proportional to the amount of initial template (i.e. telomeric sequence). The initial quantity of telomeric sequences depends on the DNA concentration and on the telomere length. To control for DNA concentration, a single copy gene (SCG), 36B4 in the initial study (Cawthon 2002), is amplified in a separate reaction and the ratio of telomere and SCG concentration (T/S) is expressed relative to a reference sample. In other words, a reference sample is used to create a standard curve for telomere and single copy gene concentration in relative units. For each sample, telomere and single copy gene concentration is estimated from the standard curves and the ratio T/S is calculated. This ratio is proportional to TL as shown by correlation with telomere restriction fragment analysis (TRF) (Cawthon 2002). Several single copy genes have been used in humans (Table 5.2), including β-2 globin (Grabowski et al 2005), GAPDH (Martin-Ruiz et al 2004) and β-actin (Koppelstaetter et al 2005). The acidic ribosomal phosphoprotein PO (36B4) gene has also been applied in mice (Callicott & Womack 2006).

Since the first introduction using a video camera and ethidium bromide (Higuchi et al 1993), real time PCR has become a well established method to quantify DNA and RNA (Bustin

& Mueller 2005). A variety of different detection chemistries have been developed (reviewed in Giulietti et al 2001; Bustin 2002), which can be divided into probe based and DNA binding dye based methods. The probe based methods follow the principles of fluorescence resonance energy-transfer (FRET, Cardullo et al 1988), involving a fluorescent dye and a quencher. Fluorescent signal can be generated by hybridization (e.g. molecular beacons) or by the 5'exonuclease activity of Taq-polymerase (TaqMan probes) (reviewed in Giulietti et al 2001). DNA binding dyes are specific to double-stranded DNA and show high fluorescence when bound, but none or low when unbound. Early studies used ethidium bromide, but today the most widely used dye is SYBR Green I. In both assay types the florescence of a PCR reaction is measured in every cycle.

As the signal is proportional to the concentration of DNA, a plot can be generated showing the course of amplification over time (cycles). A typical real-time result is shown in Fig. 5.1. In this example, a two-fold dilution of a DNA sample with seven steps, as well as one 'no template control' (NTC) was subject to real-time PCR. The amplification plots show a linear baseline region, a exponential phase of amplification and finally a plateau. A threshold fluorescent (arrow) is determined (usually by an analysis programme, which is more or less reliable (see Appendix I.IV)) where all samples are in their exponential phase and a threshold cycle (Ct value) is assigned to each reaction. The Ct is proportional to the logarithm of initial DNA quantity in each reaction and a standard curve can be constructed. The slope of the standard curve can be used to estimate the amplification efficiency of the DNA standard.

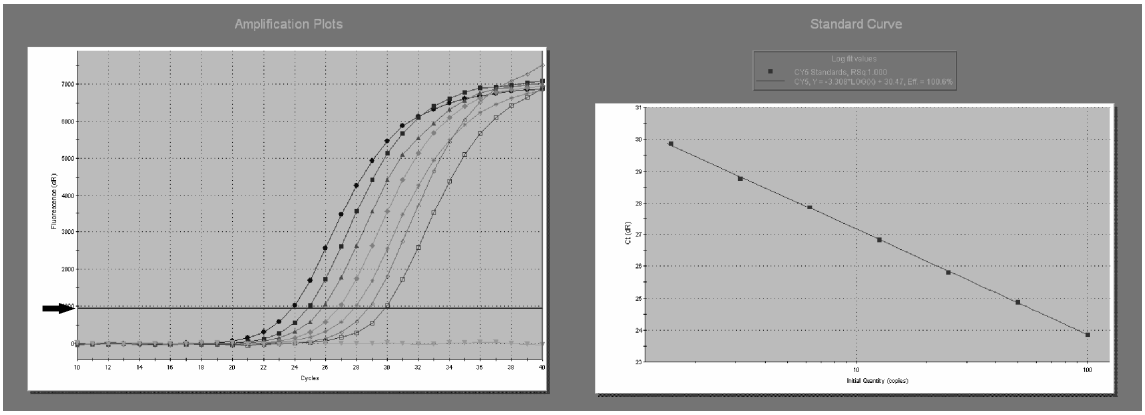

Figure 5.1: Example of a real-time PCR output. A two-fold dilution series was amplified (left) to create a standard curve (right). The arrow denotes the threshold used to calculate the threshold cycle (Ct) for each sample.

Telomere Q-PCR can only utilize DNA binding dyes because no specific fragment, necessary for probe bases assays, is amplified, but rather random pieces of telomeres. Therefore all telomere Q-PCR protocols to date used SYBR Green.

5.2 Telomere Q-PCR in kakapo

5.2.1 Method

Two single copy genes (β-actin and recombination activating gene 1 (rag1)) were tested for their usefulness as a reference gene for telomere quantitative real-time PCR in kakapo. Primers for β-actin and rag1 were designed based on available bird sequences using clustalx (Version 1.83) and pDRAW32 (Version 1.1.97). Optimization of PCR did not result in a single band of the expected length and both genes were disregarded. Instead a short sequence of the flanking region of the *Sha*1 microsatellite, previously isolated from the kakapo genome (Robertson et al 2000), was used. While this microsatellite has not been proven to be single copy, the appearance of only two alleles per individual and Mendelian inheritance supports the single copy nature of this locus. Primers were designed to amplify a 130 bp fragment excluding the microsatellite (Sha1.1f GACGCGCATGGAAGTTTTAT, Sha1.1r TTCACACATT-CCTGCTGCTG). Telomere primer sequence was obtained from R.M. Cawthon (personal

communication, Tel 1b CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT, Tel 2b GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT). The reference samples was generated by pooling 65 kakapo DNA samples. A twofold dilution series was prepared ranging from 9 ng/µl to 0.14 ng/µl (7 concentrations and one NTC) and aliquots were stored in sealed screw top tubes at -80°C.

Quantitative real-time PCR was performed in a Stratagene MX3000p real-time PCR cycler. Amplification of *Sha*1 flanking region (hereafter referred to as *Sha*1) was carried out in 20 µl FastStart SYBR Green Master (Roche) containing 75 nM each primer. Amplification profile was initial 95°C for 10 min followed by 40 cycles of 95°C for 30 sec, 61°C for 30 sec and 72°C for 30 sec. Fluorescent signal was read at the end of the annealing step and a dissociation curve was recorded at the end of the programme. Telomere amplification was carried out in 20 µl FastStart SYBR Green Master (Roche) containing 625 nM each primer. Amplification profile was initial 95°C for 10 min followed by 40 cycles of 95°C for 30 sec and 56°C for 30 sec. Fluorescent signal was read at the end of the annealing step and a dissociation curve was recorded at the end of the programme. All reactions were conducted in 0.2 ml strip tubes with 8 tubes each. Strips were sealed immediately after individual set up and stored on ice until PCR (5-30 min) to avoid evaporation during set up of the remaining tubes. Sha1 and telomere reaction were done in the same position in the cycler for the same sample. All samples were measured four times in independent PCRs on different days. Each PCR included three standard curves of the reference DNA sample in the range of 2.25 ng/µl to 0.035 ng/µl and 67 kakapo samples with an approximate concentration of 0.3 ng/µl.

To simplify calculation of relative TL with Q-PCR, the start concentration of the reference standard curve was set to 100 copies and arbitrary concentration of *Sha*1 and telomere was calculated for each sample. Telomere length was expressed as the ratio of telomere and Sha1 concentration (T/S). The mean coefficient of variation (CV) of all samples was 11.42%.

Amplification efficiency of the standard curves was 96% ± 3.7% for *Sha*1 and 86.3% ± 3.48% for telomere.

Amplification efficiency was measured for 16 samples using the standard curve method (slope of regression line) with four different concentrations of each sample (approximately 1 ng/µl to 0.13 ng/µl in two-fold dilution). In some cases (5/16 for telomere and 9/16 for *Sha*1) it was not possible to determine the efficiency from the regression line due to high scatter of the data points and the data point that resulted in the highest coefficient of determination (R^2) for the remaining three data points was eliminated. DNA quantity was estimated using four reference standard curves with virtual start concentration of 100 copies as above. Measured DNA concentration of each dilution was multiplied by the dilution factor to obtain an estimated concentration of the start sample. Measurement error for each sample was calculated as coefficient of variation (CV) of estimated DNA concentrations of all dilutions of the sample.

5.2.2 Results

Telomere length was measured from 67 kakapo using Q-PCR (Fig 5.2).

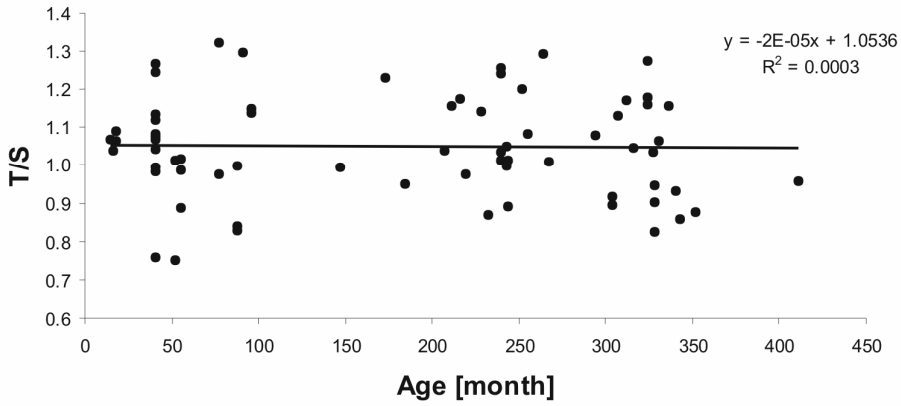

Figure 5.2: Relationship between telomere length (T/S, measured by Q-PCR) and age in 67 kakapo. There was no significant correlation (linear regression).

T/S ratio ranged from 0.75 to 1.32 and there was no correlation between telomere length (T/S) and age. Although the lack of correlation between TL and age is consistent with the TRF results (Chapter 3), there was also a lack of correlation between TL measured by TRF and Q-PCR (Fig 5.2). As the same DNA samples have been used for both assays, some form of correlation between the TRF and Q-PCR results was expected based on the earlier findings of Cawthon (2002) or Grabowski et al (2005).

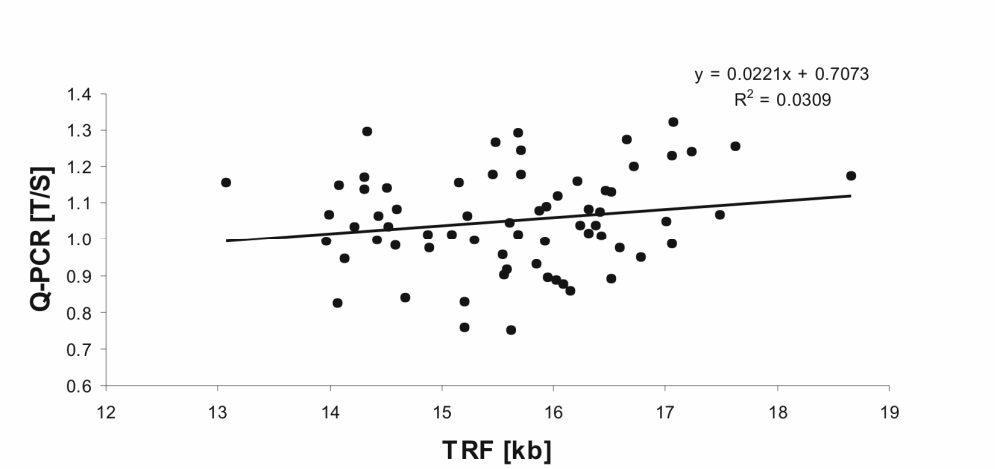

Figure 5.3: Relation between telomere length measured by Q-PCR and TRF in 67 kakapo. The same samples were measured with both methods. There was no significant correlation (linear regression).

There are two possible explanations for the discrepancy between TL measured by TRF and Q-PCR: biological or methodological errors. Biological errors can arise from interstitial telomeric repeats (ITR) that can not be distinguished from real telomeres by Q-PCR. Although birds are reported to have extensive ITR (Venkatesan & Price 1998; Delany et al 2000), there was no higher number or intensity of interstitial bands in TRF analysis of kakapo compared to fish (sea bass, but also tuna and swordfish (data not shown)) and mammals (dog and human (data not shown), see also section 2.3). Nevertheless, high amounts of small ITR, undetectable by TRF, are recognised by Q-PCR with the shortest possible telomere PCR product being 76 bp (Cawthon 2002) and could account for the difference observed. Alternatively, the normalization to DNA quantity using *Sha*1 could have failed. *Sha*1 has not been shown to be a single copy locus, although all population genetic work undertaken to date with this locus suggest, that this is

the case (Robertson et al 2000; Robertson et al 2006). The second possibility, methodological errors, leads to a more detailed examination of real-time PCR principles, particularly that of amplification efficiency.

5.3 Q-PCR Amplification efficiency

The most important variable when performing a real-time PCR is the efficiency of amplification (Pfaffl 2001; Nordgard et al 2006). Efficiency is the measure of how much of the target sequence is amplified in each cycle, with 100% or 2 (Rebrikov & Trofimov 2006) meaning a doubling of target sequence in each cycle. Both units are used interchangeably and the efficiency in percentage (X%) can by converted to the decimal number (Y) using the equation $Y = X\%/100 + 1$. As real-time quantification calculates from a threshold cycle (Ct) back to the initial amount of template DNA in the reaction (cycle 0), it is important that the amplification efficiency is the same for all samples. Curiously, most studies only optimize the standard curve to have an efficiency around 100% and do not check the efficiency of the actual samples.

The influence of efficiency differences is summarized in Table 5.1. Here, all samples start with the same amount of DNA, but differ in amplification efficiency. The values are the deviation of quantity estimate for a given efficiency relative to an efficiency of 100% (perfect doubling). The error is dependent on the threshold cycle (Ct) that the signal is recorded, because the more cycles the reaction went through before reaching the Ct, the more error is accumulated. As shown in Table 5.1, a difference in efficiency of 3% (first row, 100% - 97% = 3%) results in a measurement error of 35% when the signal is detected in cycle 20. This calculation may to some extent overestimate the error, because the amplification efficiency might change during the first cycles (Nogva & Rudi 2004) and amplification of low template concentrations might follow a stochastic course (Nogva & Rudi 2004) rather than an exponential one in the first cycles (Nordgard et al 2006). Nonetheless, this emphasizes the need to control for efficiency in each sample.

Table 5.1: Error estimation for different amplification efficiencies in Q-PCR. Error between concentration estimation of the same sample with different efficiencies relative to 100% efficiency is shown. The magnitude of the error is dependent on the threshold cycle (Ct) of detection. Error was calculated using the equation for exponential amplification ($(2^{Ct}/E^{Ct}-1)x100$).

Efficiency	**Ct 10**	**Ct 15**	**Ct 20**	**Ct 25**	**Ct 30**	**Ct 35**
1.97 (97%)	16%	25%	35%	46%	57%	70%
1.95 (95%)	29%	46%	66%	88%	113%	142%
1.90 (90%)	67%	116%	179%	260%	365%	500%
1.80 (80%)	187%	385%	722%	1290%	2260%	3900%
1.70 (70%)	408%	1045%	2480%	5710%	13000%	29500%
1.60 (60%)	920%	2740%	8570%	26400%	80700%	246400%

Reproduced and modified from 'LightCycler Relative Quantification Software Version 1.0', (Roche 2001).

Telomere real-time PCR is especially vulnerable to efficiency errors, because two independent reactions (telomere and single copy gene) are used to estimate the relative telomere length, both potentially including efficiency errors. Different thresholds for acceptable efficiencies have been suggested (e.g. 90%-110% ('Introduction to Quantitative PCR', Startagene 2004), 93%-105% (Nolan et al 2006)). These limits are only indicative, as the actual values are of minor importance if all samples show the same efficiency. As most studies do not check the efficiency of each sample, it is assumed that there is a higher chance of consistency between the standard and sample efficiency if the standard is optimized to a value around 100% (the theoretical perfect amplification). Cawthon reported an efficiency of 80%-100% for his standard curves used in the telomere real-time PCR assays (personal communication).

The efficiencies of *Sha*1 and telomere standard curves where reproducible between experiments on different days (*Sha*1 96% ± 3.7% and telomere 86.3% ± 3.48%, n=4). The lower efficiency for telomere amplification can be explained by mismatches of the telomere primers, which were introduced to prevent primer dimer formation (Cawthon 2002). Primer mismatches have previously been shown to reduce amplification efficiency in Q-PCR (Smith et al 2002). However, other studies reported telomere amplification efficiencies of 100.5% (Nordfjall et al 2005, deduced from supplementary figure) and 107.8% (Gil & Coetzer 2004, calculated from slope of standard curve). These discrepancies might be due to different analysis parameters,

which strongly influence the magnitude of amplification efficiency estimated by the standard curve method (Appendix I.IV).

To test if the kakapo samples had a similar efficiency to the standard curves, 16 samples were tested using the standard curve method. The efficiency for the reference DNA standard curves was 105.2% for *Sha*1 and 78.4% for the telomeric amplification (Fig. 5.4) and the R^2 was 0.997 and 0.996, respectively. The efficiency of the samples ranged from 89.4% to 129.9% for *Sha*1 and from 65.8% to 108.3% for telomere. To assess the influence of efficiency on quantification the start concentration was calculated for each dilution step (i.e. measured concentration by dilution factor) and a coefficient of variation (CV) was calculated from the 3-4 dilutions for each sample.

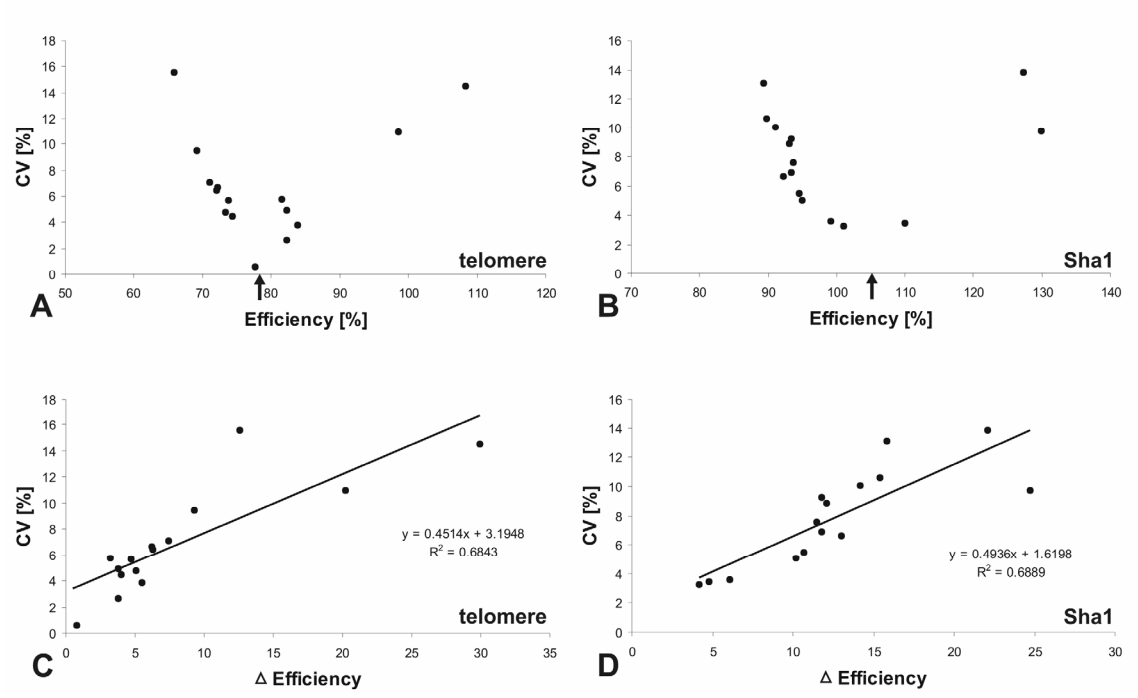

Figure 5.4: Effect of amplification efficiency on coefficient of variation (CV). CV was calculated from 3-4 dilutions of each sample and efficiency was estimated from the slope of the regression line. (A+B) CV against sample efficiency and (C+D) against difference between sample and standard curve efficiency (Δ Efficiency). Efficiency of the standard curve is denoted with an arrow.

The error introduced by efficiency is dependent on the detection cycle (Ct, Table 5.1), thus higher dilutions with a later Ct should accumulate more error. Consequently, different dilutions of the same sample show a higher standard deviation and CV if they have a higher error

associated with efficiency. Fig 5.4 shows that the CV for samples is higher the more their efficiency departs from the efficiency of the standard curve. Efficiencies of the same sample for telomere and *Sha*1 reaction were not correlated. As telomere Q-PCR uses the ratio of telomere and single copy gene quantification, the error of both reactions is incorporated into the final calculation of telomere length. The confounding effect of efficiency could explain the lack of correlation between telomere length observed by TRF and Q-PCR in kakapo.

5.4 Discussion

5.4.1 Telomere length of kakapo

The measurement of telomere length using Q-PCR was confounded by considerable variations in amplification efficiency. It is not clear what caused this variation in the case of kakapo. Different efficiencies are generally a sign of PCR inhibition. To ensure sufficient quantity of DNA for necessary replications and further studies a pool of 65 kakapo DNA samples was used as the reference sample. This reference should have the average concentration of PCR inhibitors present in all DNA samples, which might explain the spread of sample efficiencies to both directions of the reference efficiency (Fig. 5.4). Unfortunately, each assay can only be optimized for one sample (typically the reference sample) as the PCR conditions must be the same for all reactions to obtain reliable results. Consequently, it is not possible to improve the results by re-optimizing PCR conditions, as they were already optimal for the reference sample. The DNA was extracted using a Phenol-Chloroform method (Sambrook & Russell 2001) and appeared to be of high quality and purity based on agarose gel electrophoresis and absorption spectrum analysis by Nanodrop ND 1000 (Nanodrop Technology). Alternative extractions using a Qiagen DNeasy Blood & Tissue Kit did not reduce the range of efficiencies (data not shown).

5.4.2 Effect of amplification efficiency

As the exact cause of the error remains unknown, the consequences are worrying. Efficiency error is not a random error on the sample level. It depends on the starting concentration that determines the number of cycles the sample must go through to reach the fluorescence threshold. The more cycles needed, the more error accumulates. While efficiency tends to be reproducible, such that a sample of a given concentration can be measured several times with the same result, the result is still wrong if the efficiency is significantly different from that of the standard curve. The only way to detect efficiency error is to measure different concentrations of each sample and calculate back to the initial quantity using the dilution factor or estimate efficiency from the individual amplification curve of each sample (Smith et al 2002). Unfortunately this quality control step is rarely included in real-time studies and is especially not in telomere real-time assays.

Of 20 randomly chosen publications using real-time PCR (Appendix Table II.II), only one mentions the efficiency of the standard curves (Gil & Coetzer 2004). Two others show a figure including the slope of the standard curves (Nordfjall et al 2005, supplementary figure; Zhang et al 2007) that can be used to deduce efficiency. Cawthon indicated an efficiency of 80%-100% on request (personal communication). While the efficiencies obtained by Gil & Coetzer (2004, 96% SCG, 108% telomere) and Nordfjall et al (2005, 106.7% SCG, 100.5% telomere) and probably the one from Cawthon (2002) were in an acceptable range, the amplification efficiency of the telomere reaction reported in other studies is far beyond the acceptable range. For example, a recent study by Zhang et al (2007) examining TL in tumour and adjacent non-tumour tissue shows an efficiency of 330% for the telomere standard curve. No reliable measurements can be made in this range. Furthermore, approximately half of the threshold cycles (Ct) for samples in Zhang et al (2007) are outside of the range of the standard curve, ignoring the basic principle of real-time PCR (Nolan et al 2006). It is especially worrying

to see this kind of mistakes made in medical studies where researchers are potentially dealing with health and lives of patients (T. Horn, in press).

Such mistakes are symptomatic of the increasing trend of scientists becoming users rather than investigators. Companies advertise the ease with which real-time PCR assays can be developed and applied and disregard the absolute need to control each single amplification plot (Nolan et al 2006) and parameters, such as amplification efficiency (Bustin 2002; Bustin & Nolan 2004; Bustin et al 2005). Analysis of gene expression is automated to an unreliable extend ('You just tell the program which reference gene you use and put in the reactions for different treatments and the program gives you a ready-to-publish graph', BioRad salesman when setting up a new real-time PCR cycler in our School).

Of the remaining 17 telomere Q-PCR publications selected, the quality of their results cannot be assessed due to the lack of basic information such as amplification efficiency of the standard curves. Unfortunately, when asked at a Q-PCR meeting, 31% of participants did not consider it necessary to indication amplification efficiency in publications (Bustin 2005). Despite that, it would be expectable that amplification efficiency is at leased mentioned in a publication investigating the effects of tissue fixing agents on telomere Q-PCR (Koppelstaetter et al 2005), especially if the reference DNA is a unfixed sample. These agents are obligatory PCR inhibitors as they are designed to stop any enzyme activity in the fixed cells. Likewise a test of efficiency would be appropriate when measured telomere length increases from approximately 4kb to approximately 9kb within 3.7 years in a elderly human patient (Martin-Ruiz et al 2005), an observation contradicting all we know about telomere length change in humans.

Special caution should be taken if the reference DNA is not derived from one of the test samples, or a pool of several samples, but is rather derived from a commercially available standard (Martin-Ruiz et al 2004) or cell line sample (Nordfjall et al 2005; Grabowski et al 2005). In this case, an equal efficiency to the actual samples can not be assumed as potential inhibitors are not shared. Fehrer et al (2006) suggested the use of cell lines as a standard for

telomere Q-PCR. Although amplification efficiency is not mentioned in the publication, the slopes of the standards curves of three different cell lines show, that one of them exhibits a highly divergent efficiency. Clearly, the choice of cell line used to create the standard curve would have a high influence on the estimated telomere lengths of the assay. The telomere lengths of a sample calculated from two standard curves with different amplification efficiencies will inevitable be different. Furthermore, the error introduces in not linear (i.e. higher error for samples with higher Ct, see Table 5.1), so that the distribution of TL for the whole dataset is altered.

5.4.3 Choice of single copy gene

Another potential error source is the choice of single copy gene and the primers selected to amplify that fragment. Primer sequences of single copy genes employed for telomere real-time PCR in humans can be tested using the UCSC *in-silico* PCR (http://www.genome.ucsc.edu/cgi-bin/hgPcr) on whole genome builds (Table 5.2).

According to the human NCBI Build 36.1 reference sequence (March 2006), the most frequently used primer pair, for 36B4, amplifies two fragments of the same length located on different chromosomes (chr 2 and chr 12, Table 5.2). Furthermore, one of these fragments does not have a known function and might not be conserved among individuals. The same applies to a primer pair for GAPDH used by Koppelstaetter et al (2005), although the additional fragment belongs to a known gene and might be present in all humans. Interestingly, one primer pair used in several tissues of humans (Thibeault et al 2006) did not show any *in-silico* amplification in the human genome, but would amplify the 36B4 homolog Arbp in mouse (NCBI Build 37).

Table 5.2: *In-silico* PCR of primers used to amplify single copy genes. SCG = single copy gene

SCG	sequence	fragment length [bp]	chromosome	gene	used by
36B4	CCCATTCTATCATCAACGGGTACAA CAGCAAGTGGGAAGGTGTAATCC	75 75	12 2	36B4 unknown	(1-7)
36B4	TAAAGACTGGAGACAAGGTGGGAG AGAAAGCGAGAGTGCAGGGC	?†	?†	?†	(8)
GAPDH	GACTCCACGACGTACTCAGC AAATCCCATCACCATCTTCC	220 116£	12 5	GAPDH ADRA1B	(3)
GAPDH	GACTCCACGACGTACTCAGC AAATCCCATCACCATCTTCC	210	12	GAPDH	(9)
β2-globin	GCTTCTGACACAACTGTGTTCACTAGC CACCAACTTCATCCACGTTCACC	120	11	β2-globin	(10-11)
β-actin	AAAGACCTGTACGCCAACAC GTCATACTCCTGCTTGCTGAT	331	7	β-actin	(3)

† these primers were not found to amplify in humans, but a 166 bp fragment of the 36B4 homolog in mouse; £ allowing one nt mismatch at the 5'end of one primer. For references see Appendix II.II.

Although whole genome builds are prone to assembly errors and *in-silico* PCR is not a substitute for PCR, a more careful evaluation of single copy genes and their primers should be desirable. Some of the used genes (GAPDH, β2-globin and β-actin) are housekeeping genes commonly used for expression analysis. Housekeeping genes are supposed to have a constant expression level in the cell, but that does not necessarily mean that they are single copy.

5.4.1 Correlation between TRF and Q-PCR

A final source of methodological error involves conversion of relative telomere length from T/S values to actual length in kb. This is necessary to compare results between studies using different methods to measure TL. Table 5.3 shows that the relation between T/S and the real telomere length is not constant among studies. Although most studies found a high correlation between results obtained by Q-PCR and TRF, the conversion factors are different. Adoption of a conversion formula from another study, therefore, would result in highly inaccurate estimates for real TL in kb. Still, some of the published conversion factors are of

unknown origin (Thibeault et al 2006; Njajou et al 2007, Table 5.3), and questionable as they miss one of the two parameters (slope and intercept of the regression) necessary to convert T/S to kb.

Table 5.3: Correlation between telomere length measured by TRF and Q-PCR.

relation TRF-(T/S) [bp]	**R^2**	**n**	**found by**	**formula also used by**
y=1910.5x+4157	0.677	95	Cawthon (2002)	Cawthon et al (2003)
y=3198.9x+3128	0.818	43	Grabowski et al (2005)	Nordfjall et al (2005)
y=1406.1x+8685	0.664	N/A ≈ 10	Callicott & Womack (2006)	
y=1095.4x+6846	0.766	N/A	Harris et al (2006)	
factor 4270, no reference	-	-	Njajou et al (2007)	
factor 2870[†]	-	-	Thibeault et al (2006)	

[†] the source of this value is not clear; the authors description implies they calculated their own regression, but the sample size and age range do not match their study, but do match Cawthon (2002). Nevertheless the conversion factor does not fit the regression by Cawthon (2002).

All these examples highlight that there are numerous and widely-ignored error sources even in the supposedly established telomere Q-PCR assay. In this study one of them, namely varying amplification efficiency, was identified in the experiments undertaken. Therefore one have to be cautious about the utility of any interpretation based on the results obtained here for kakapo telomere length. It is not clear what caused the high efficiency fluctuations, but they led to the investigation of possibilities to correct for different amplification efficiencies in real-time PCR assays using the kakapo *Sha*1 locus as a model system (Chapter 6).

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6. Correction for amplification efficiency in real-time PCR

This chapter introduces inhibitors as the main cause of varying amplification efficiency in real-time PCR and presents an experimental approach to correct for PCR inhibition.

6.1 Introduction

The effects of PCR inhibition on amplification efficiency have been known since the invention of PCR (e.g. Innis et al 1990; reviewed in Wilson 1997). Early methods to quantify nucleic acids, like competitive PCR relied on a similar amplification efficiencies (hereafter termed only efficiency) for both, sample and competitor (reviewed in Zimmermann & Mannhalter 1996, see below). The invention of real-time PCR enabled the investigation of PCR dynamics in greater detail. Higuchi et al (1993) suggested that real-time PCR could be used to study the influence of PCR inhibitors and to identify ways to avoid them. Unfortunately, PCR inhibition is still a widely neglected field with few reviews (e.g. Wilson 1997; Radstrom et al 2004) that gathered information reported by various studies as a by-product of their original research. Although amplification inhibition affects all fields of study that use PCR, it is of major concern in molecular diagnostic and gene expression analysis.

Real-time PCR is currently the "gold standard" for quantifying nucleic acids. It is used in most fields of biology, including ancient DNA research, population genetics, sex identification, and forensic (for references see Morin et al 2007). Another important application of real-time PCR is diagnostic, where it is used to detect pathogens or the presence of genetic modification vectors in food samples, microorganisms in soil samples or microbiological infections in clinical samples (reviewed in Radstrom et al 2004). Diagnostic real-time PCRs are highly dependent on the detection limit as they indicate the presence or absence of the target DNA or RNA from a sample. When screening for targets with low abundance, like hepatitis B in blood donor samples (Drosten et al 2000) or *Salmonella* in food samples (Reynisson et al 2006), slight changes in

amplification efficiency can lead to false negative results with potentially disastrous consequences for the tested individual (e.g. clinical PCR, blood screening) or group of individuals (e.g. food screening). While errors introduced by amplification efficiency in biological applications may not be as disastrous, they might also have important consequences for research results and their application (e.g. estimation of wrong population structure or sex bias).

Another field that that is dominated by real-time PCR is the analysis of gene expression. Here, real-time PCR is currently the standard for verification and quantification of microarray results. To measure the level of mRNA, an additional step, a reverse transcription (RT), is necessary to obtain quantitative results. Although this field of study suffers from several methodological difficulties, including variability in extraction and reverse transcriptase efficiencies and choice of reference gene or external standard (reviewed in Freeman et al 1999; Bustin 2000; Stahlberg et al 2005), amplification efficiency is also a major source of error in gene expression analysis (Bustin 2002; Peirson et al 2003; Bustin et al 2005). In contrast to diagnostic PCR, where the main focus lies on identifying false negatives, for expression studies methods to correct for different efficiencies had to be developed. Despite this need, no universal solution to that problem has been found and amplification efficiency and PCR inhibition is widely ignored (Bustin 2005; Nolan et al 2006b).

6.1.1 PCR inhibition

A variety of substances have been identified to interfere with PCR amplification. They derive from the samples themselves, the extraction procedure, or from upstream reactions like reverse transcription. Others are inherent to PCR reagents or conditions (see below).

Although written more than ten years ago, the most comprehensive review of PCR inhibitors is that by Wilson (1997). Wilson gathered information on inhibition of PCR from all kinds of studies and reported measures to facilitate amplification in the presence of these

inhibitors. Back then, as is still the case today, the mechanism of inhibition for most inhibitors is unknown. Several mechanisms have been discussed by Wilson (1997) and Radstrom et al (2004), which include inactivation or blocking of the polymerase and the degradation, capturing or blocking of DNA. The identification of several substances commonly used in sampling and DNA extraction (e.g. heparin, SDS, phenol, ethanol, EDTA, Wilson 1997) has emphasised the need for highly purified DNA for reliable real-time PCR. The need for highly purified DNA is tempered by the observation that damaged DNA inhibits PCR (Sikorsky et al 2004), which prohibits the use of some of the more rigorous extraction methods.

Sample independent factors that are specific to laboratories or assay types can also inhibit PCR. Different instruments (Reynisson et al 2006), polymerases (Radstrom et al 2004; Wolffs et al 2004), plastic ware (Chen et al 1994) or batches of PCR buffer (Burgos et al 2002) can all contribute to variations in Q-PCR efficiency between laboratories. Rigorous primer and probe design together with stringent optimization of PCR conditions is also essential to obtain optimal efficiency (Reynisson et al 2006).

6.1.2 Correction methods for PCR inhibition

6.1.2.1 Mathematical corrections

Several mathematical models have been developed to estimate the efficiency of individual amplification curves and correct the quantification of nucleic acids accordingly (Nordgard et al 2006). Most of these models are based on a linear regression of the exponential part of the amplification curve (e.g. Liu & Saint 2002; Tichopad et al 2002; Peirson et al 2003), but contradictory results have been reported in these studies. For example, Ticopad et al (2002) found that their approach introduced more error than the use of a fixed efficiency for all samples, but other authors claimed a superior quantification accuracy (i.e. how well an estimate approaches the real value) using their models (Liu & Saint 2002; Peirson et al 2003). A

systematic study investigating error propagation of mathematical models for efficiency correction concluded that all models tested introduced more random error (reduced precision, usually measured as standard deviation of repeated estimates) than they gain in accuracy (Nordgard et al 2006). This random error is believed to be due an inability to estimate efficiency with the necessary precision (Peirson et al 2003), because small derivations from the true efficiency are amplified exponentially in mathematical models (Nordgard et al 2006).

6.1.2.2 Experimental corrections

Experimental corrections are based mainly on the concept of competitive PCR (reviewed in Zimmermann & Mannhalter 1996; Freeman et al 1999). These assays utilize the simultaneous amplification of the target DNA and a competitor in a single reaction to estimate the absolute quantity of the target DNA in a sample. The competitor is designed to have the same primer binding sites as the target, but possesses a modified internal sequence that allows the amplified target and competitor to be distinguished and quantified using agarose or capillary gel electrophoresis or HPLC. Usually a dilution series of the competitor is amplified with a constant amount of target and a standard curve from the quantification ratio (target/competitor) and the competitor concentration is constructed (Fig. 6.1). As target DNA and competitor compete for polymerases and primer in the reaction, it is assumed that the concentration of both is equal at a target/competitor ratio of one.

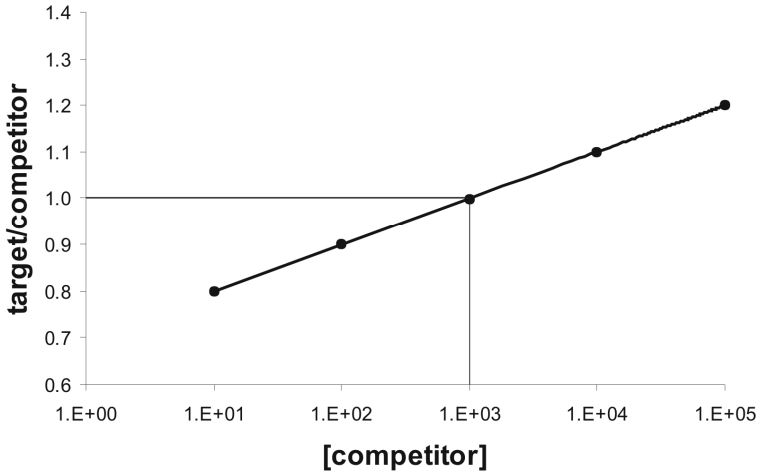

Figure 6.1: Quantitative competitive PCR. Target and competitor are co-amplified and compete for PCR reagents. Different reactions with constant target and decreasing competitor concentration are subject to PCR and the ratio of both fragments is plotted against initial concentration of the competitor. When the ratio is one, an equal start amount of target and competitor DNA is assumed.

Competitive PCR quantification relies on an equal amplification efficiency for both reactions (Freeman et al 1999). This assumption has been justified by the use of a competitor that has the same primer binding sides and only minimal sequence difference to the target (Freeman et al 1999). With the introduction of quantitative real-time PCR (Q-PCR), competitive PCR lost support in favour of the real-time PCR standard curve assays or normalization of gene expression to reference genes. Competitive PCR now mainly exists in the form of internal controls in diagnostic PCR to help identifying false negatives (Drosten et al 2000; Burggraf & Olgemoller 2004; Burggraf & Olgemoller 2005; Hodgson et al 2007).

Competitive PCR, however, can be combined with RT real-time PCR (Grove et al 2006). These authors designed an RNA competitor for a piscine nodavirus quantification assay. Four different concentrations of the competitor were co-reverse transcribed and co-amplified for each sample using standard RT real-time PCR. The absolute viral load in the sample was calculated through regression of the Ct ratio ($Ct_{tagret}/Ct_{competitor}$) and the initial competitor concentration similar to Fig. 6.1. The authors demonstrated a high sensitivity and linearity for their approach. In theory, competition should correct any inhibition during RT and real-time PCR, but tests were

not done in the present of inhibitors. Efficiency was close to 100% in all real-time PCR reactions suggesting that no inhibition occurred in the validation reactions for this assay. A major drawback of this method is the need to prepare four reactions (including RNA extractions and RT) for each sample, which significantly adds to the time and money invested in the assay.

The usefulness of competitors has also been demonstrated by Tani et al (2007a; 2007b). Instead of real-time PCR they used the ratio of fluorescent probe bound to the target or the competitor after conventional PCR or loop-mediated isothermal amplification. They demonstrated that this approach successfully corrected for inhibition by urea, humic acid and triton X-100 (Tani et al 2007b), but the precision of the quantification is somewhat compromised at the ends of the rectangular hyperbola obtained as standard curve.

Here, a novel protocol combining competitive PCR and real-time PCR was developed to correct for differences in efficiency observed between reactions, with the goal to improve reliability of telomere real-time PCR in kakapo (see Chapter 5).

6.2 A simplified model of efficiency correction

Although based on competitive PCR, the here resented model assumes an independent amplification of target and an internal standard oligonucleotide (ISO). In practice this can be achieved by adding sufficient primer and polymerase for both amplifications to not limit each other. The amount of DNA in each PCR cycle can be calculated by the formula (Rebrikov & Trofimov 2006):

$$N_C = N_0 \bullet E^C \qquad \text{Equation 6.1}$$

where N_C is the amount of DNA after cycle C, N_0 is the start amount and E is the amplification efficiency. Using equation 6.1, the amount of DNA after each cycle during PCR can be calculated for different efficiencies (Fig. 6.2A). As the amount of DNA is proportional to the signal intensity in real-time PCR, these graphs can be used to simulate the first cycles (background and exponential phase) of a real-time PCR and a threshold cycle (Ct) can be

calculated. Standard curves using different initial quantities are shown for several efficiencies in Fig. 6.2B. A difference between 90% and 110% efficiency, the most commonly accepts maximum variation, already results in a 5 fold error in the estimated start concentration when detected at cycle 15.

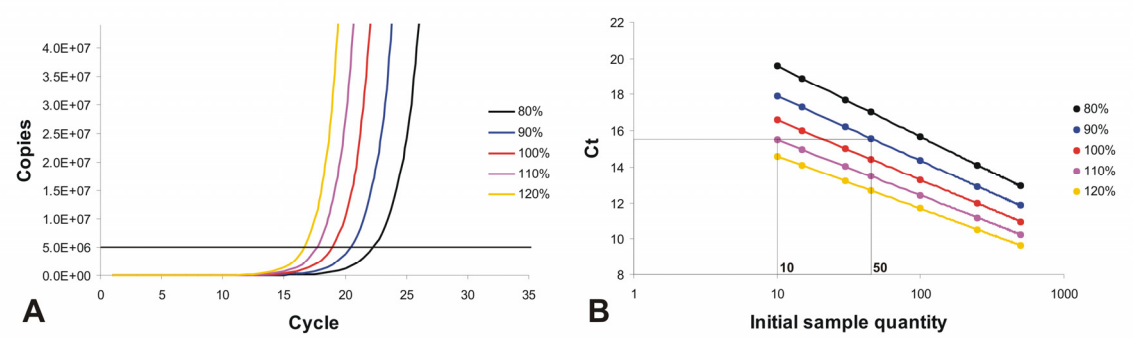

Figure 6.2: Simulation of real-time PCR. (**A**) Accumulation of DNA per cycle calculated with equation 6.1 for a start quantity of 10 copies of DNA with different amplification efficiencies. A threshold can be introduced to calculate Ct values. (**B**) This Ct values are then used to create standard curves for different efficiencies. The vertical line shows the difference between 90% and 110% efficiency for a given Ct resulting in a 5 fold difference in estimated DNA concentration.

A simultaneous amplification of an ISO can be added to this calculation (Fig. 6.3A). As SYBR Green must be used for the telomere reaction (Chapter 5) and cannot distinguish between amplification of sample and ISO, a third variable, the sum of target and ISO, was introduced. The resulting Ct values are shown in Fig. 6.3B. The initial quantity of ISO was kept constant while the quantity of the sample was increased.

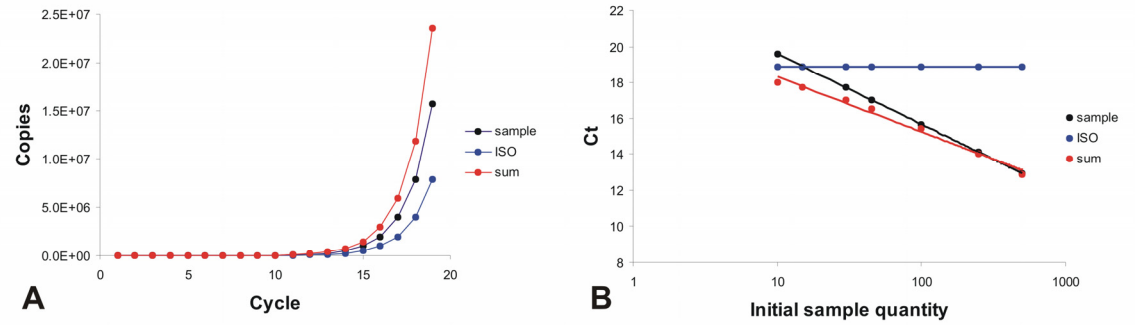

Figure 6.3: Simulated amplification of sample, ISO and sum of both. (**A**) Amplification curves. (**B**) Standard curves, the initial quantity of ISO was kept constant while that of the sample was increased.

In this system the Ct of ISO accounts for efficiency and the Ct of the sample or the sum accounts for the initial sample concentration. Dividing the Ct of the sample or sum by the Ct of ISO (S/ISO ratio) results in a parameter that incorporates initial quantity and efficiency. Fig. 6.4 shows the resulting standard curves for different efficiencies using the S/ISO ratio.

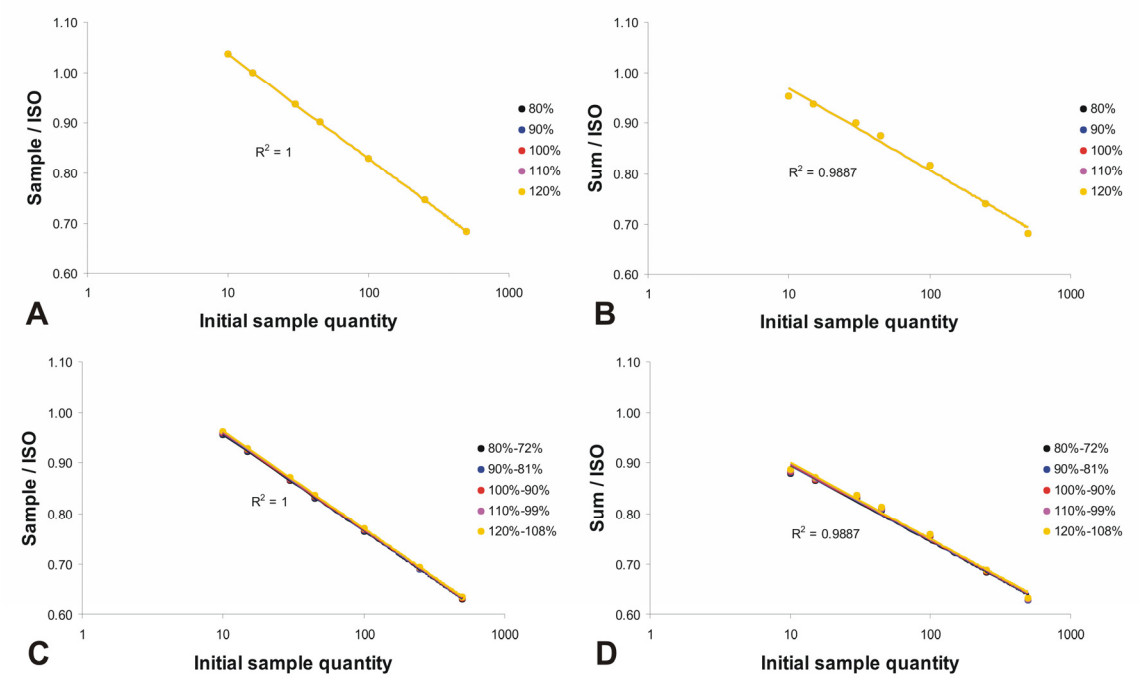

Figure 6.4: S/ISO ratio for standard curves of different efficiencies. The initial quantity of IS was constant for all reactions. (**A**+**B**) The same efficiency was assumed for sample and standard. Only the last regression line is visible, because the S/ISO correction results in the same values for all efficiencies. (**C**+**D**) The efficiency for sample and standard was different, but changed proportional to each other, first number is efficiency of sample, second of ISO.

A central assumption of competitive PCR is that target and competitor share the same efficiency. Based on this assumption, the Sample/ISO ratio results in a perfect correction of efficiency with matching values for all efficiencies (Fig. 6.4A). If we lower the stringency and allow different efficiencies for sample and ISO, but assume that they change proportional to each other (i.e. both amplifications get inhibited equally), there is still a high level of agreement between the estimates for the different efficiencies (Figure 6.4C). Although the Sum/ISO gives slightly worse coefficient of determination than the Sample/ISO ratio (Fig. 6.4B+D), it is still a good fit to the expected values. In theory, the Sample/ISO and Sum/ISO ratios should be able to correct for the influence of efficiency as well as for small differences between reactions caused

by uneven temperature distribution in the thermocycler block and fluctuations in the fluorescence light source or detection system.

6.3 Experimental design

6.3.1 Primers and probes

Internal standard oligonucleotides (ISO) were designed for the kakapo Sha1 microsatellite locus (Robertson et al 2000) and telomeres (Cawthon 2002). Both oligonucleotides shared the primer binding sites with the sample, but had a binding site for a TaqMan probe (TaqRep) in the middle. To decrease the possibility of non-specific binding of the probe, the internal ISO sequence was designed based on a study by Hampikian & Andersen (2007), who developed a program to search for absent oligonucleotide sequences in GenBank. They created a dataset with short sequences (primes) that have not been found in any living organism to date. Following suggestions by Proudnikov et al (2003) TaqMan probes were designed to possess an internal quencher to increase the sensitivity of the probes. The sequences of primers and probes are shown in Table 6.1. All probes were phosphorylated at the 3' end to inhibit extension by polymerase. ISOs and probes were synthesised by Sigma-Proligo (The Woodlands, TX, USA).

To test if the complexity of the internal standard has an influence on the inhibition of its amplification, the ISO for the single copy locus (ShaRep) was inserted into the plasmid pJet1 using GeneJet^{TM} PCR cloning kit (Fermentas Life Sciences). The plasmid containing ShaRep (named ShaPlas) was prepared by Andrew Bagshaw (University of Canterbury) using a continuous CsCl-ethidium bromide gradient (Sambrook & Russell 2001). The plasmid was then restricted with *EcoRI* and purified from low-melting-temperature agarose gel (Sambrook & Russell 2001).

Table 6.1: Primers and probes used for experimental efficiency correction.

Primers	
Sha1f	GACGCGCATGGAAGTTTTAT
Sha1r	TTCACACATTCCTGCTGCTG
Tel 1b	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT
Tel 2b	GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT
ISOs	
ShaRep	GACGCGCATGGAAGTTTTATGATAGCCTGCTCTACA**CTCGTACGTTACGGATCGCGGTTGCT AG**AGAAAAGATATATTTTCTGGTGTGAAGCATCCTTCCCAGCAGCAGGAATGTGTGAA
TeloRep	AGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTA**CTCGTACGTTACG GATCGCGGTTGCTAG**GGGTTGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTT
TaqMan probes	
TaqSha	Cy5-CATGTTCATCT(BHQ2)ATGCAGTCTGTCACCAG-Phosphate
TaqRepI	Hex-CTAGCAACCGC(BHQ1)GATCCGTAACGTACGAG-Phosphate
TaqRepII	Rox-CTAGCAACCGC(BHQ2)GATCCGTAACGTACGAG-Phosphate

Underlined letters are primer binding sites, letters in bold are the probe binding site, BHQ is Black Hole Quencher. TaqSha binds to genomic Kakapo DNA and TaqRepI + II to the ISO's.

6.3.2 Inhibitors

EDTA, which chelates $Mg2^{+}$ ions necessary for polymerase function, was used to partially inhibit the amplification reaction and modulate efficiency for assay optimization. Inhibition was dose dependent and reproducible. Additional inhibitors used were sodium dodecyl sulfate (SDS), heparin, urea and ferric chloride ($FeCl_3$). The inhibition concentrations are given in Table 6.2.

Table 6.2: Concentration of inhibitors.

Inhibitor	**Concentration range**
EDTA	0 - 2.19 mM[†]
SDS	0.05 - 0.08 g/l
heparin	0.016 - 0.125 U/ml
urea	100 - 160 mM
$FeCl_3$	12 - 24 µM

[†] depending on master mix

6.3.3 Real-time PCR

Real-time PCR was performed in a Stratagene Mx3000P or Mx3005P and data were analysed with MxPro software (Mx3005P V.3.00 Build 311, Schema74, Stratagene). All reactions were conducted in 0.2 ml strip tubes with 8 tubes each. Strips were sealed immediately after individual set up and stored on ice until PCR (5-30 min) to avoid evaporation during set up of the remaining tubes. Three different master mixes (MM) were used: FastStart SYBR Green master mix (Roche), TaqMan universal PCR master mix (ABI) and a house made master mix based on Taq-Polymerase (Bioline) containing 2.35 mM MgCl, 160 µM each dNTP and 0.04 U/µl polymerase. The reaction volume was 25 µl. Only the Sha1 locus and its internal standards (ShaRep and ShaPlas) were used to test the efficiency correction method. Primers Sha1f and Sha1r were used at 200 pM, all TaqMan probes were used at 50 pM and ISO ShaRep was used at 0.8 fM. Concentration of ShaPlas was not measured due to low yield from the purification process and optimal quantity for correction experiments was determined by dilution series. SYBR Green I (Invitrogen) was added to ABI and Bioline MM to a concentration between 0.12 and 0.48 fold, depending on the master mix. No non-specific amplification was observed by melting curve analysis of reactions containing SYBR Green. All experiments were conducted with the same DNA sample, a DNA pool of 50 bird samples, which were purified using phenol-chloroform extraction (Sambrook & Russell 2001, two phenol-chloroform steps, one chloroform step). After ethanol precipitation, DNA strings were transferred to TE without centrifugation to avoid co-precipitation of damaged DNA. The DNA pool was further purified using a Qiagen DNeasy Blood & Tissue Kit according to manufacturers instructions.

To simplify calculations of inhibition, the relative DNA amount of the standard curve was designated as 100 units diluted down two-fold in seven steps. The real amount of the pooled DNA sample was 37.5 ng diluted down two-fold.

6.3.4 Inhibition and efficiency

The effect of EDTA on efficiency and DNA quantification was tested first. Exact estimation of efficiency is difficult (Peirson et al 2003; Nordgard et al 2006) and is highly dependent on the baseline and threshold settings. Amplification- and baseline-derived thresholds change depending on the selected wells and might create distortion when comparing efficiencies obtained if only a single standard curves is selected in the analysis program (see Appendix I.IV). Therefore, default amplification-based threshold settings were used to estimate the threshold value for the whole experiment (all reactions selected) and this threshold was applied to all reactions of the experiment.

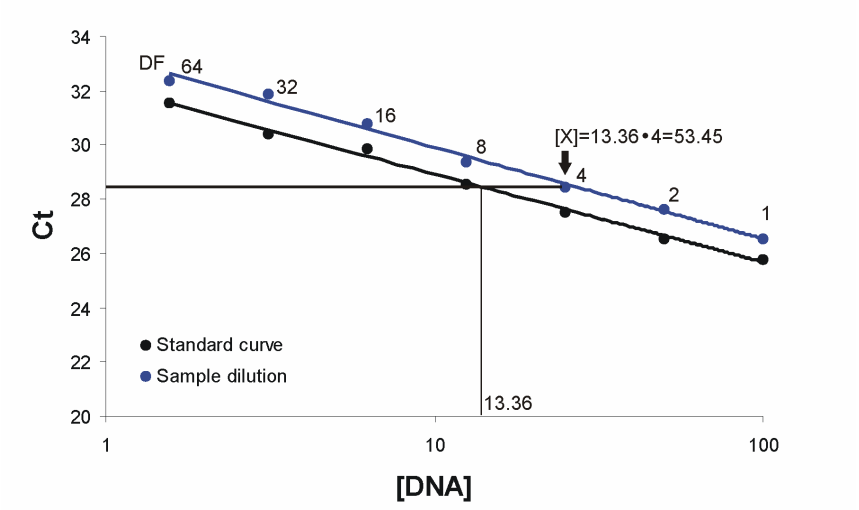

Figure 6.5: Calculation of mean effect of inhibition. A standard curve with no or little inhibition is used to estimate DNA concentration for each reaction of a sample dilution series. For sample X the concentration estimated by the standard curve is 13.36 and sample X is the third of a two-fold dilution series and has therefore a dilution factor (DF) of 4. The initial concentration for sample X is therefore 13.36•4=53.45. The initial concentration was calculated for each point of a dilution series and averaged. The average initial concentration of the standard curve is 100, as it has been defined as having a concentration of 100 (mean of 100x1; 50x2; 25x4; etc).

The susceptibility of the different reaction mixes to EDTA inhibition is shown in Fig. 6.6. All reactions show a shift towards higher Ct with increasing EDTA concentrations. The ABI master mix showed the highest tolerance with a minimum EDTA concentration of 1.25mM necessary for inhibition. To quantify the effect of inhibition, the estimated DNA concentration was calculated for each reaction relative to non- or minimally inhibited samples. Therefore, the

curve with the lowest EDTA concentration was used as the standard curve and all other reactions were treated as unknown samples. The initial DNA concentration can be calculated from the standard curve and the dilution factor of each sample. For each dilution series the average DNA concentration and standard deviation was calculated. The default concentration of the dilution series used as the standard curve is 100, because the DNA concentration of undiluted standard was defined as 100 (section 5.2). An example of the calculation is shown in Fig. 6.5.

The mean initial concentration decreased in all reaction mixes with increasing EDTA concentration. While the mean concentration shows the accuracy of the estimate (difference to 100), the standard deviation represents the precision of the measurement.

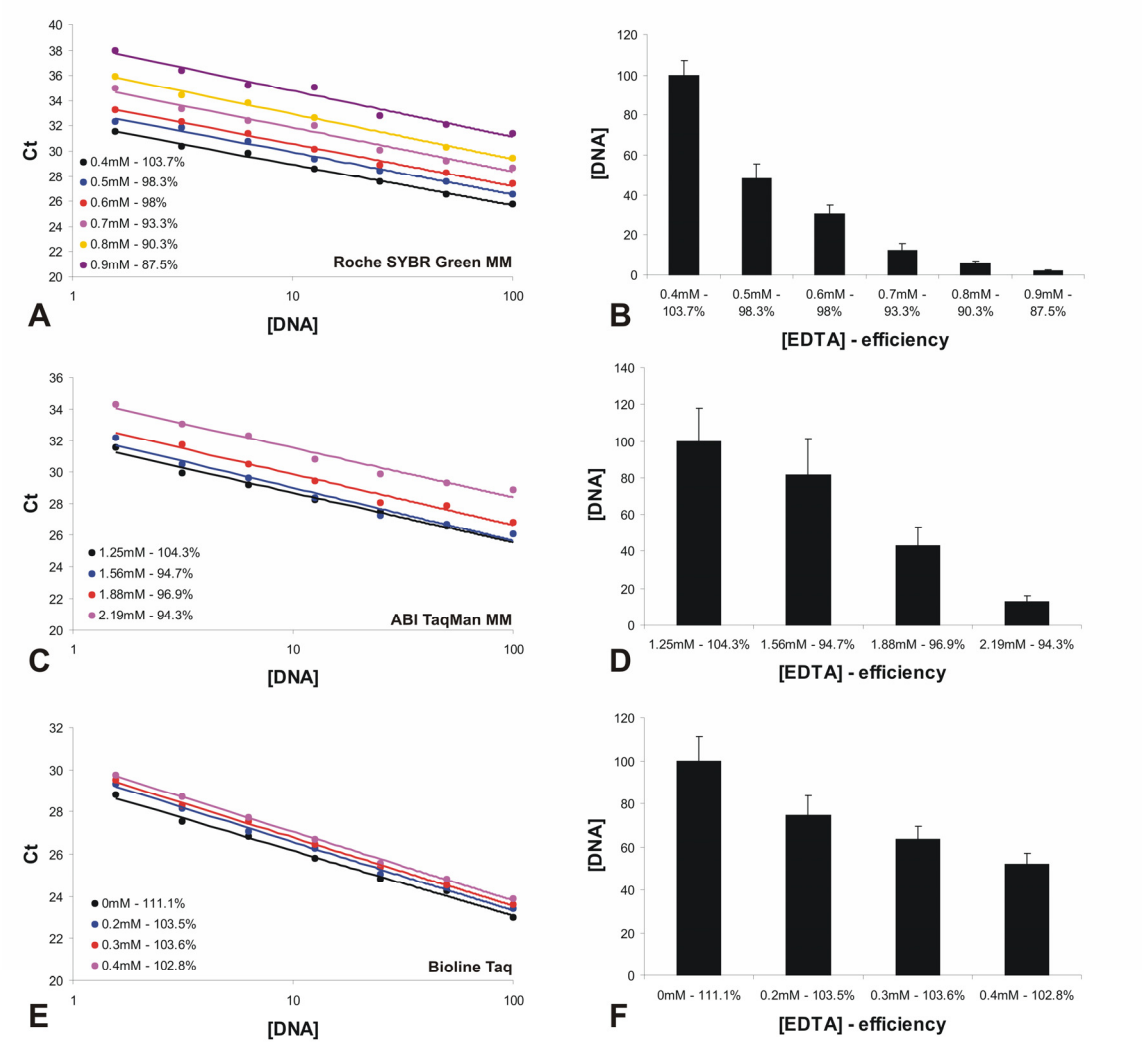

Figure 6.6: Effects of EDTA on different master mixes. Dilution series in the presence of various concentrations of EDTA were measured (**left**) and mean initial concentration ± SD was calculated as described in Fig. 6.5 (**right**). Amplification was recorded with SYBR Green in **A**/**B** and **C**/**D** and with TaqSha in **E**/**F**.

Efficiency slowly decreased with increasing EDTA concentration in Roche SYBR Green MM, but not the ABI TaqMan MM or Bioline Taq. The exact estimation of amplification efficiency is difficult, but this difference between the MM was reproducible. However, the influence on estimated DNA concentration in the samples was similar with an increase in EDTA concentration leading to an increasing underestimation of quantity in all master mixes (Fig. 6.6 right). In these experiments the amount of inhibitor was kept constant for different DNA concentrations (i.e. each sample of the standard curve).

In practice, when using a reference sample to create a standard curve, the inhibitor is co-diluted with the DNA. Figure 6.7 shows how a synchronous dilution of DNA and inhibitor influences the amplification efficiency in the Bioline MM. Three dilution series were prepared with the same DNA: 1) DNA diluted with H_2O, 2) DNA diluted with EDTA solution (final concentration 0.4 mM in PCR reaction) and 3) DNA spiked with EDTA (0.4 mM in PCR) and diluted with H_2O (i.e. DNA and EDTA diluted). The dilutions with H_2O (1) and EDTA (2) showed a similar efficiencies (100%-103%), while the dilution with DNA and EDTA both diluted with H_2O (3) had a much higher efficiency (128%). The standard curve of 3 starts with a similar Ct as 2 for the highest DNA concentration, but gradually approaches curve 1 as the influence of EDTA diminishes in further dilution steps. This shows that inhibitors can increase the amplification efficiency measured by the standard curve method.

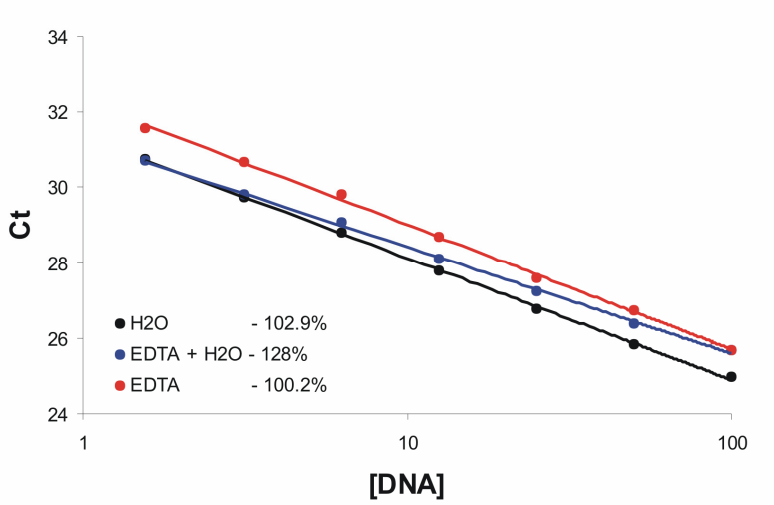

Figure 6.7: Effects of inhibitors on amplification efficiency estimates. The same amount of DNA was diluted with H_2O or EDTA or spiked with EDTA and diluted with H_2O (EDTA + H_2O). EDTA concentration in the PCR reaction was 0.4 mM for EDTA curve and 0.4 mM diluted down two fold for EDTA + H_2O curve.

6.4 Efficiency correction

6.4.1 Different master mixes

The amplification of target DNA was measured using SYBR Green or the TaqMan probe TaqSha. Although SYBR Green simultaneously measures target DNA and ISO amplification (see Section 6.2), it is the only method for correction of telomere Q-PCR, as no specific binding site for a TaqMan probe exists within the telomere amplicon. A more precise correction should be obtained by sample detection using TaqMan probe (Fig 6.4). Both methods were tested in different master mixes.

The main condition of the efficiency correction method is an independent amplification of genomic DNA and the internal standard oligonucleotide. The Roche SYBR Green MM failed to provide enough amplification capacity for independent amplification of target DNA and ISO for most DNA concentrations. This might be caused by a limited amount of polymerase or simply the fact that this master mix is optimized for SYBR Green and not for the use of TaqMan probes.

The ABI TaqMan MM on the other hand is optimized for TaqMan probes and does not contain SYBR Green. Careful optimization of primer, probe and added SYBR Green concentrations resulted in an efficiency correction shown in Fig. 6.8. The amplification of ISO (dye Hex) had a constant Ct while the SYBR Green Ct changed with target DNA concentration in non-inhibited reactions (Fig. 6.8 A). The divergence of standard curves obtained from target DNA alone with different concentrations of EDTA could be minimized when corrected with ISO amplification (compare Fig. 6.8 B and C). However, the standard deviation, derived from the whole standard curve as described in Figure 6.5, was very high indicating high variability in this setup. Surprisingly, the replacement of SYBR Green with TaqSha to measure amplification of target DNA only did not work with the ABI TaqMan MM, although it is optimized for reactions including two TaqMan probes (e.g. SNP assays).

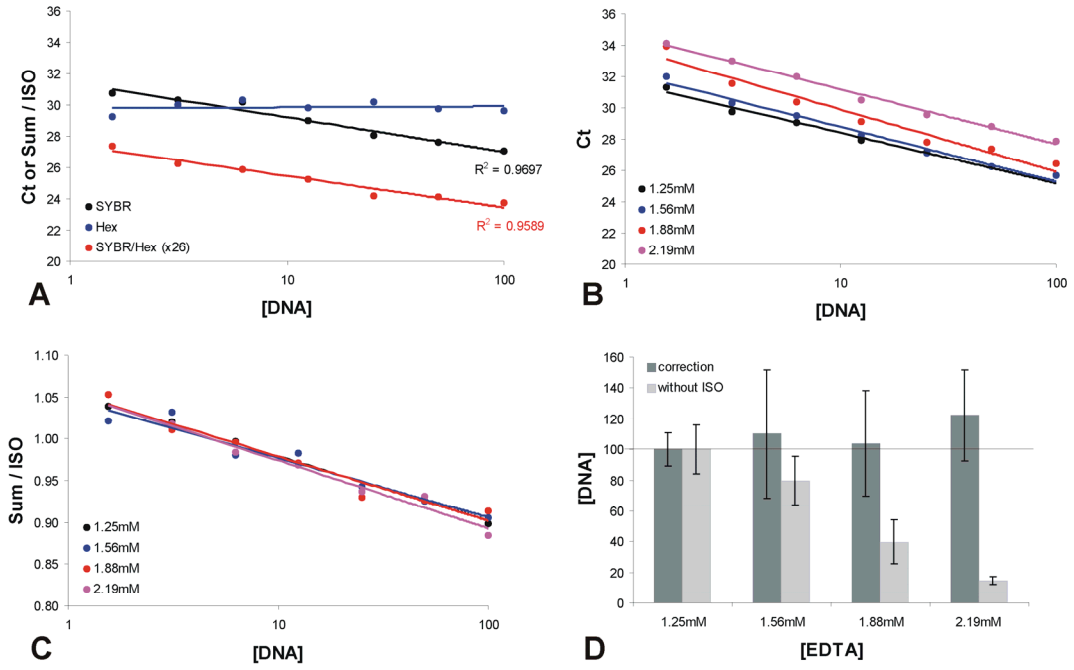

Figure 6.8: Inhibition correction using ABI TaqMan MM and SYBR Green. (**A**) Standard curves for total amplification (SYBR) and ISO (Hex) and the correction ratio Sum/ISO (multiplied by 26 to fit in range) in non-inhibited reaction. Note the constant Ct for ISO indicating an amplification independent from target DNA. (**B**) Effect of EDTA on target DNA amplification in a reaction without ISO and (**C**) corrected from a reaction with ISO. (**D**) Estimated mean target DNA concentration without and with correction for different amounts of EDTA.

The house made Bioline master mix proved to be best for the detection of two TaqMan probes (TaqSha for target DNA and TaqRep for ISO) simultaneously (Fig 6.9 + 6.10). In this experimental setup, the correction can be compared to the same reaction without the ISO (termed 'sample separately') or to the amplification of target DNA within the correction reaction (termed 'sample uncorrected'). While the first one represents the results obtained by conventional real-time PCR, the second one represents the real effect of inhibition, corrected for in the same reaction. Although 'sample uncorrected' is more appropriate to evaluate the potential of the correction method within the system, 'sample separately' shows how well the system works compared to standard real-time PCR that ignores inhibition, as presented in most publications. In this study, the same reactions was always run with and without the internal standard oligonucleotide (Fig 6.9A+B). In theory both measurements of target DNA should be the same, as DNA and ISO amplify independently, but slight differences might arise from additional reagents (TaqRep) or the total amount of nucleic acid present in the correction reaction. These differences were small compared to the effect of EDTA (Fig. 6.9D).

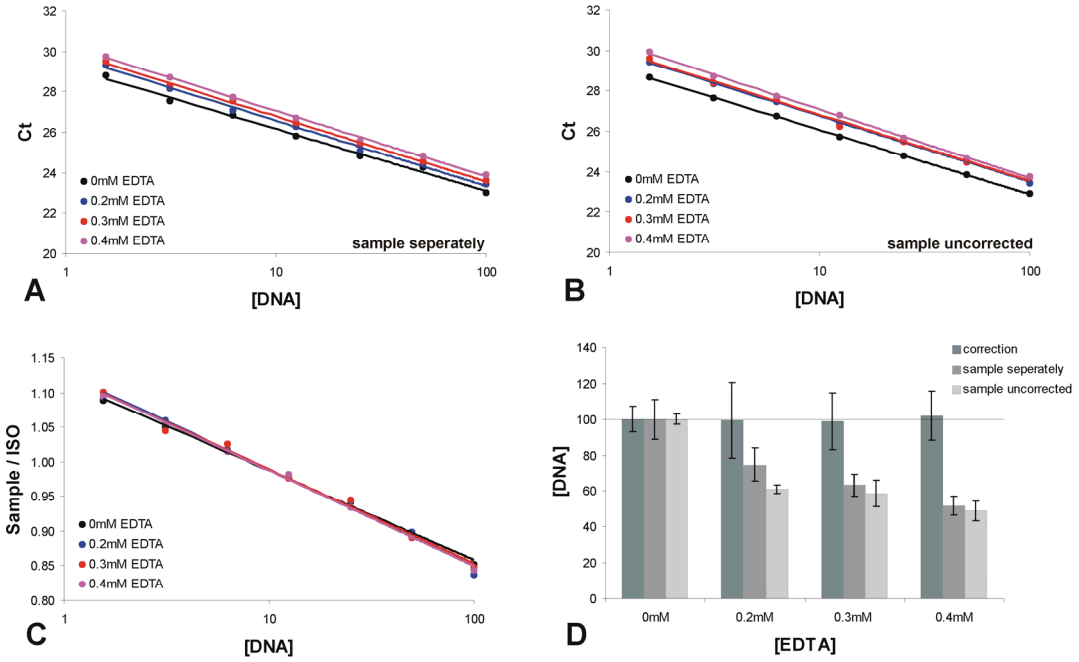

Figure 6.9: Inhibition correction using Bioline Taq MM with both TaqMan probes, moderate inhibition. (**A**) Standard curves for target DNA amplification from a setup without ISO and (**B**) with ISO. (**C**) Correction of inhibition using the ratio of sample and ISO Cts and (**D**) estimated mean target DNA concentrations for different EDTA amounts.

Both, 'sample separately' and 'sample uncorrected', showed shifts in the standard curves in response to EDTA (Fig. 6.9A+B). The correction using ISO eliminated most of this shift (Fig 6.9C+D). Although the standard deviation was still elevated, the corrected estimate is highly accurate for EDTA ranges of 0.2-0.4 mM EDTA (Fig. 6.9) and 0.4-0.8 mM EDTA (Fig. 6.10). The lower standard deviation of the uncorrected DNA estimates indicate a higher precision, but the DNA quantity reveals a high degree of inaccuracy.

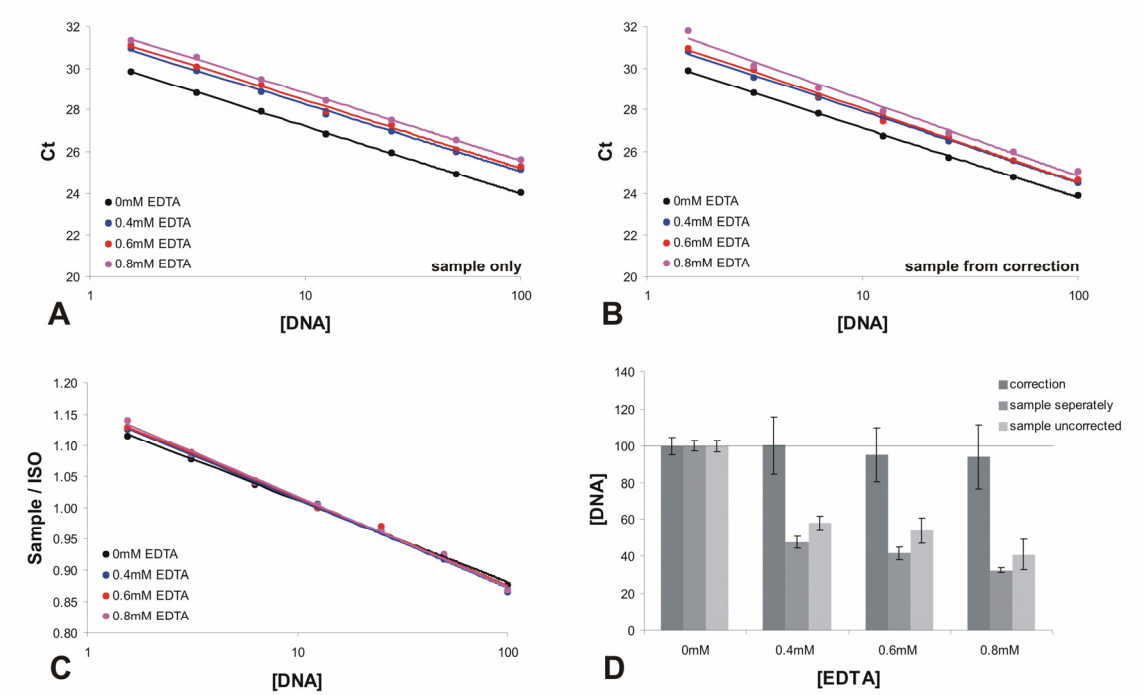

Figure 6.10: Inhibition correction using Bioline Taq MM with both TaqMan probes, high inhibition. (**A**) Standard curves for target DNA amplification from a setup without ISO and (**B**) with ISO. (**C**) Correction of inhibition using the ratio of sample and ISO Cts and (**D**) estimated target DNA concentrations for different EDTA amounts.

6.4.2 Different inhibitors

As the Bioline Taq MM gave the best results for EDTA inhibition correction, it was also used to test the correction method with other inhibitors. SDS and heparin were chosen because they are chemicals frequently used in blood sampling. SDS is present in most lysis buffers and suspected to reduce accuracy in avian diagnostic PCR (Freed & Cann 2006), while heparin is commonly used as an anticoagulant for blood samples and is known to inhibit PCR (Satsangi et al 1994; Abu al-Soud & Radstrom 2001). Urea and $FeCl_3$ were chosen as representatives of

sample intrinsic inhibitors. Urea is an important inhibitor found in wastewater (Tani et al 2007b) and faeces (Wilson 1997) and iron, a degradation product of haemoglobin, is suspected to be one of the factors responsible for PCR inhibition by blood (Wilson 1997; Abu al-Soud & Radstrom 2001). Triton X-100, phenol, haemoglobin and sucrose did not inhibit PCR in the concentrations tested (≤0.1%; ≤0.1%; ≤ 200 µg/ml; ≤ 30mM, respectively).

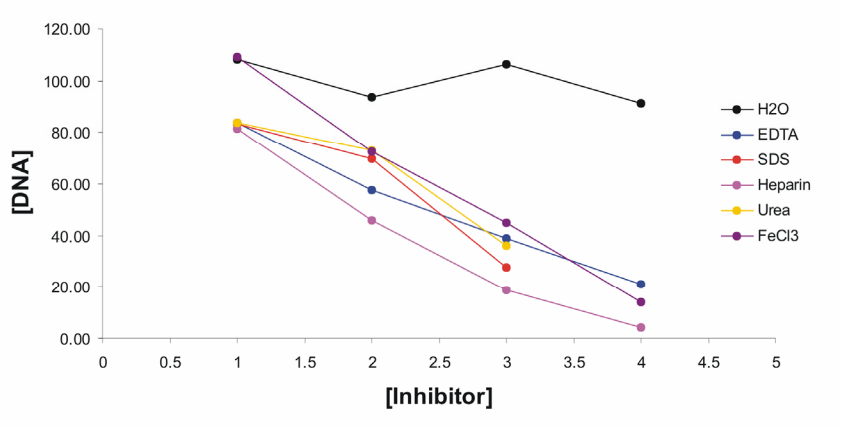

Figure 6.11: Inhibition of Bioline Taq master mix by different inhibitors. All reactions contained a virtual DNA concentration of 100. No ISO was present in this setup. Concentrations of inhibitors are shown in Table 6.2 and Figure 6:13. Missing points indicate a total inhibition.

The four additional inhibitors showed strong effects on PCR amplification in the Bioline Taq MM when uncorrected (Fig. 6.11). The effect of these inhibitors was also tested in Roche SYBR Green and ABI TaqMan MM without any ISO (Fig 6.12).

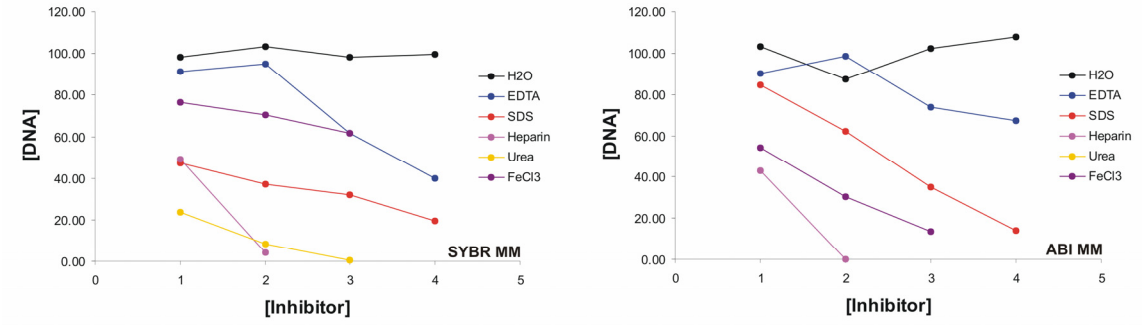

Figure 6.12: Inhibition of Roche SYBR Green and ABI TaqMan MM by different inhibitors. All reactions contained a virtual DNA concentration of 100 units. No ISO was present in this setup. Concentrations of inhibitors are shown in Table 6.2 and Figure 6:13. Missing points indicate a total inhibition.

The correction method was tested with the additional inhibitors using water and EDTA as controls. Figure 6.13 shows the estimated concentration for "sample uncorrected" and sample corrected by ISO amplification from the same tube. A separate reaction without ISO showed similar results as "sample uncorrected", but was not included because the magnitude of inhibition differed between reactions. Inhibition of target DNA amplification was generally slightly lower in the correction reaction, probably due to some "blocking" effect of the additional ISO in these reactions (data not shown). Therefore the correction should be compared to the target DNA amplification in the same tube rather than to a reaction with a slightly different composition. Water was included as a standard of uninhibited PCR (virtual DNA concentration 100) and EDTA as a positive control for the correction.

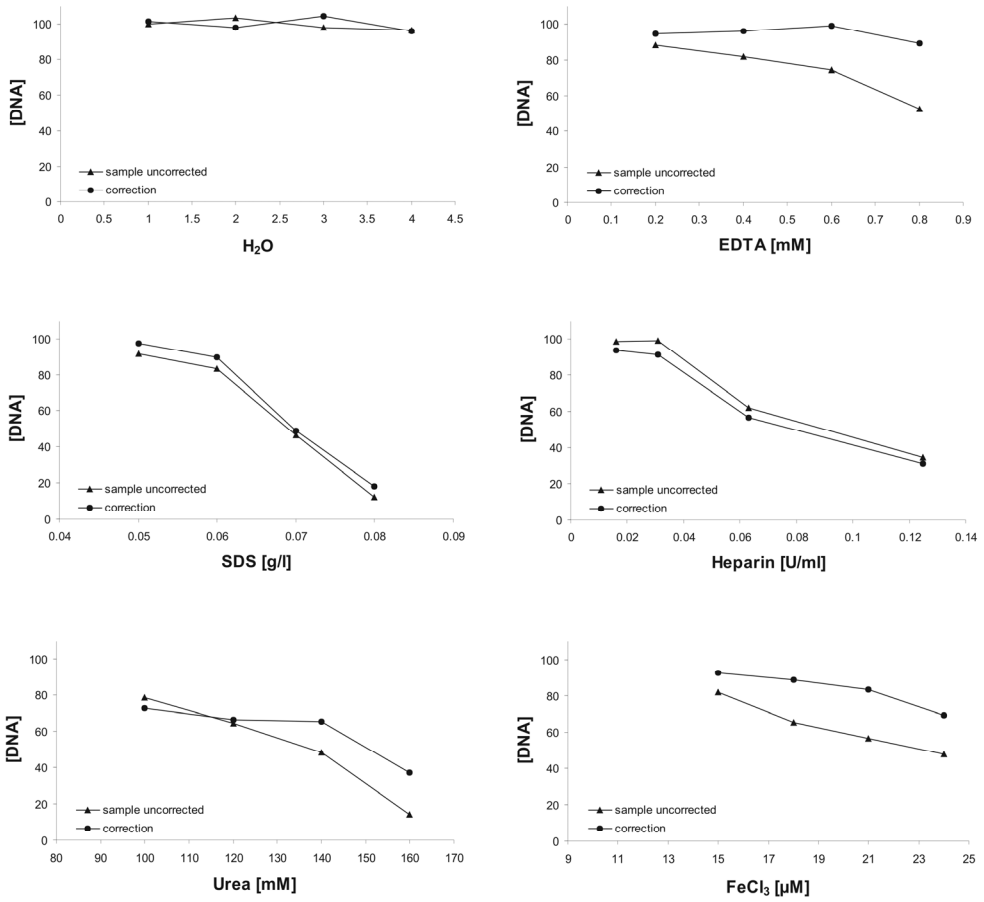

Figure 6.13: Correction of different inhibitors using ShaRep. Four reactions with H_2O were used as a standard without inhibition. The effect of EDTA, and partly of FeCl3, inhibition on DNA quantification could be corrected using this approach, but all other inhibitors were resistant to this correction method.

The H_2O reactions showed a high precision for the "sample uncorrected" and the correction ratio (Fig. 6.13). The coefficient of variation was with 3.7% for uncorrected and 3.88% for corrected estimation very low. As expected, EDTA inhibition was corrected with a high accuracy and precision (74.24 ± 15,61 vs. 94.75 ± 4.24 corrected). All other inhibitors could only be partially corrected at best, with the effects of some inhibitors unable to be corrected (Fig. 6.13). The amplification of ISO responded differently to the inhibitors than did amplification of the target DNA. Figure 6.14 shows the inhibition curves for EDTA and heparin. The Ct of sample and ISO increased at the same rate with increasing EDTA concentration such that the ratio stayed constant. In contrast, the amplification of ISO was not affected by heparin while target DNA amplification was heavily inhibited (down to 25% of real concentration estimated at 0.125 U/ml heparin). The amplification pattern of ISO in respect to inhibitors did not change when sample DNA was excluded from reaction (data not shown).

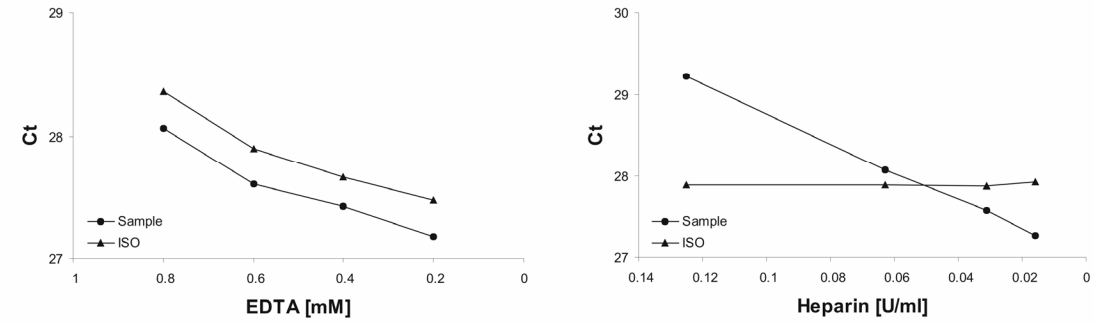

Figure 6.14: Inhibition curves for EDTA and heparin. Cts of Sample and ISO change proportional for different concentrations of EDTA, while heparin only inhibits the amplification of the sample, but not of the ISO.

As both, the target sequence and ISO share the same primer pair and have similar length, similar inhibition seems to be a logical expectation. One possible explanation of different susceptibility to inhibitors might be accessibility of template in the first cycles of PCR. While the ISO is freely available and easily denatured, the target sequence in the sample DNA is flanked by other sequences and might be subject to secondary structures. To test the influence of

complexity of DNA on PCR inhibition, the ISO was inserted into a plasmid (ShaPlas) and the influence of inhibitors on this level of DNA organization was tested.

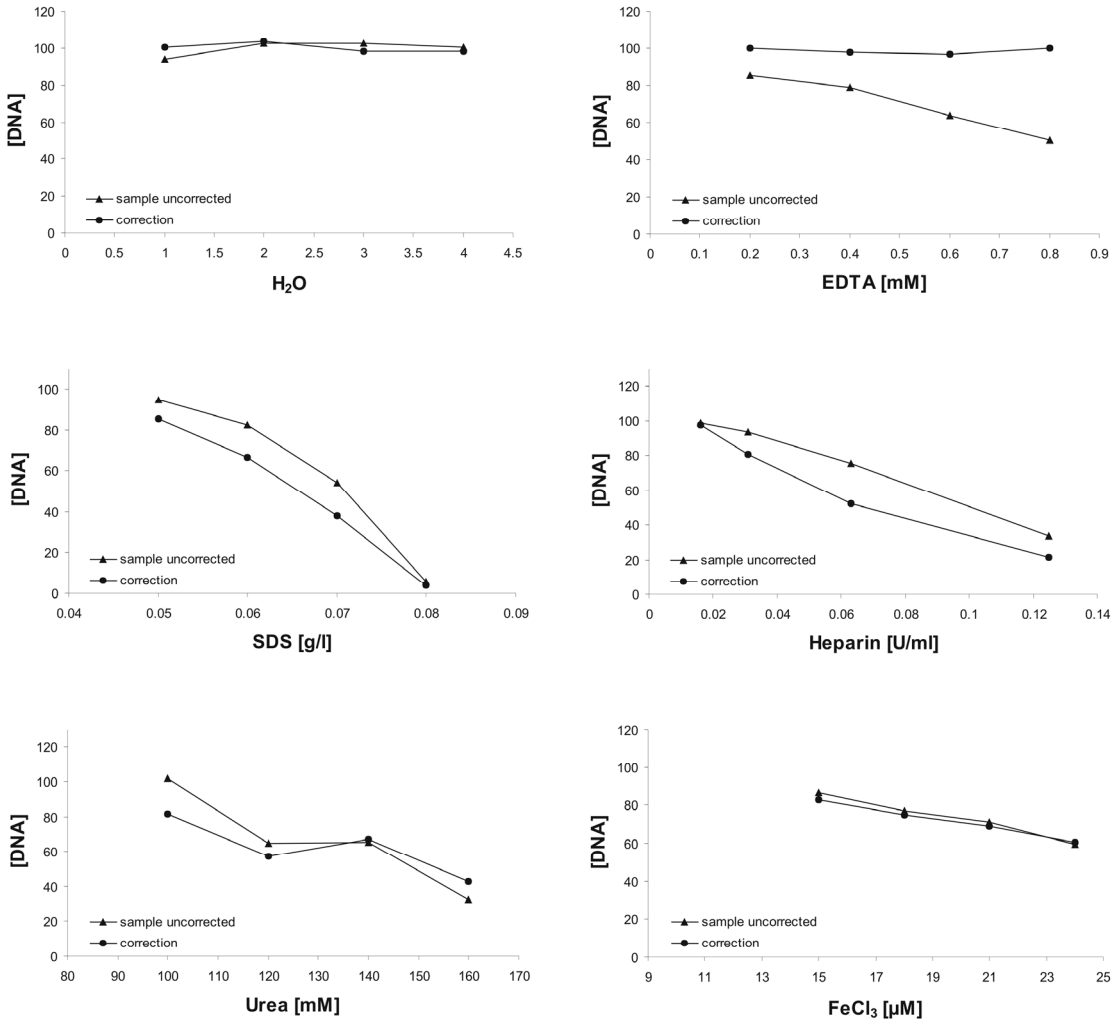

Figure 6.15: Correction of different inhibitors using ShaPlas. Four reactions with H_2O were used as a standard without inhibition. The effect of EDTA inhibition on PCR amplification efficiency could be corrected for using this approach, but all other inhibitors were resistance to this correction method.

The use of ShaPlas instead of ShaRep did not improve the correction of inhibition (Fig. 6.15). Inhibition curves for ShaPlas were similar to the ones from ShaRep, and ShaPlas showed the same immunity against inhibition as ShaRep when target DNA was absent.

6.5 Discussion

The importance of amplification efficiency in real-time PCR assays is still highly underrated. Repeated warnings to evaluate efficiency in quantitative and diagnostic real-time PCR and suggestions to including efficiency values in any publication (Bustin 2002; Bustin & Nolan 2004; Bustin et al 2005), along with the emphasised importance of efficiency by manufactures of real-time PCR cyclers (e.g. Table 5.1), are widely ignored by the scientific community. Misciting of manuals adds another layer to the problem. According to Gil & Coetzer (2004), an efficiency of 49%-121% is stated as acceptable in an older Roche lightcycler manual. Those values could not be confirmed by Roche NZ (personal communication) and are far outside the suggested range (e.g. 93%-105%, Nolan et al 2006a).

Although the invention of real-time monitoring eased investigation of the effects of amplification efficiency and inhibitors on PCR (Higuchi et al 1993), our knowledge about inhibition of PCR is still marginal (Radstrom et al 2004). The telomere Q-PCR assay in kakapo identified a strong correlation between efficiency and accuracy (Chapter 5). The more the efficiency of a sample differed from the efficiency of the standard curve, the more inconsistent were the estimations for different dilutions of the same sample. The intriguing feature of efficiency error is that it has a high precision. Repeated measurements will show a high agreement as the error is inherent to the reaction and hence is reproducible. Nevertheless, the quantification obtained by a number of repeated measurements can be highly inaccurate, even if the standard deviation is very low (Fig. 6.10).

Internal standard oligonucleotides have been used mainly for diagnostic PCR to identify false negatives (Drosten et al 2000; Stocher et al 2003; Burggraf & Olgemoller 2004; Hodgson et al 2007). For quantification of nucleic acids by competitive PCR several different concentrations of ISO must be co-amplified with the each sample (Freeman et al 1999; Grove et al 2006). This is not only time consuming and resources demanding, but might also be impossible if sample

DNA quantity is limited. The present study showed that a simple model, which combines an internal amplification standard with the competitive PCR approach, can be used to correct for the effects of different amplification efficiencies in Q-PCR. The need to amplify the same sample several times is eliminated by replacing the Ct of conventional real-time PCR measurements with the ratio of sample Ct and ISO Ct. A normal standard curve assay can then be used to estimate the DNA concentration corrected for efficiency relative to a reference sample. An absolute quantification is also possible if the concentration of the reference sample is known.

The here presented correction method was able to correct inhibition of EDTA with high accuracy, but low precision using a combination of SYBR Green and a TaqMan probe. High accuracy and precision was achieved using a system with two TaqMan probes. However, this approach failed to correct for the influence of several other potentially common inhibitors (SDS, heparin, urea and $FeCl_3$) in DNA samples extracted from blood. Findings of the present study highlight the need for a re-evaluation of some common assumptions of PCR efficiency, inhibition and the use of internal standard oligonucleotides in Q-PCR.

6.5.1 Inhibition of different master mixes

Inhibitors are believed to reduce the amplification efficiency by decreasing the polymerase activity or the availability of DNA or primers (Wilson 1997). Ironically, inhibition can be detected by elevated efficiency (>100%) measured by the standard curve method (Fig. 6.7). To obtain a standard curve, a dilution series must be constructed in which the inhibitor gets diluted together with the DNA. Consequently, the inhibition is higher in the first steps of the dilution series and decreases with increasing dilution, thus the Ct of high DNA concentration will be higher than expected, thereby decreasing the slope of the standard curve, thus leading to a higher estimate of amplification efficiency.

To investigate the influence of an inhibitor on efficiency, its concentration must be kept constant for the whole dilution series of DNA. In this way each reaction gets inhibited equally

assuming an independence of DNA concentration and inhibition. That this assumption might be invalid is shown in the comparison of EDTA inhibition in different master mixes (Fig. 6.6). DNA amplification using the Roche MM is inhibited gradually and the efficiency decreases inversely to the EDTA concentration. This is consistent with the expected pattern shown in Figure 6.2. All curves point to a virtual intersect where the DNA concentration is so high that it meets the fluorescence threshold without amplification (Ct = 0).

A different pattern is revealed by experiments using the two other master mixes. Here, efficiency does not change for the three highest EDTA concentrations of the ABI or Bioline MM. The standard curve is not tilted, but rather shifts towards a higher Ct. This pattern indicates that the whole dilution series started with a lower DNA concentration, which is not the case as the same dilution series was used for all curves. Depending on its concentration, EDTA seems to exclude a certain percentage of the DNA available for amplification from each step of the dilution series. Alternatively, it may delay the start of the amplification process by a number of cycles, perhaps in a stochastic manner.

The effect of EDTA was independent of the detection chemistry, as the ABI MM used SYBR Green and the Bioline MM a TaqMan probe. In practice, the different reaction of standard curves to EDTA should not cause any problem, because extrinsic inhibitors in the sample are still recognizable by increased efficiency (Fig. 6.7) and intrinsic inhibitors such as contamination of the reaction mix or the plastic of the tube, act on the standard curve and the samples in the same way. Nevertheless, it questions our understanding of PCR amplification and inhibition. EDTA is a chelator that binds Mg^{+} ions and therefore inhibits polymerase and also reduces specificity of primers (Khosravinia & Ramesha 2007). Both mechanisms can not easily explain the observed pattern. Further investigation of this phenomenon should increase our understanding of PCR and the processes taking place in the first cycles.

6.5.2 Correction of EDTA inhibition

All master mixes underestimated the target DNA concentration in response to increasing EDTA concentrations (Fig. 6.6), but the main condition for the correction method, independent amplification of target DNA and ISO, was only met by ABI and Bioline MM (for an example, see Fig. 6.8A). The best results for a mixed detection system in the presence of EDTA were obtained with the ABI MM. Here, SYBR Green was used to measure the combined amplification of target DNA and ISO, and the TaqMan probe TaqRepI was used to detect amplification of ISO only. This setup must be used when a TaqMan probe for the target DNA can not be designed, as is the case for telomere amplification (Cawthon 2002).

Seven different dilutions of the same sample were measured to obtain an indication of the working range of the correction method. The mean of the seven dilutions was a much better estimate of the real concentration than the measurement without correction (Fig. 6.8D). However, the standard deviation (SD) obtained from these measurements comprised 25-40% of the estimated DNA concentration. There was no pattern within the different DNA concentrations of a dilution series, indicating that the error causing the high SD is random and cannot be avoided by choosing a certain concentration range of target DNA. Although the correction method achieved superior accuracy over non-corrected estimates, the precision is very low, making repeated measurement necessary for reliable estimation of DNA concentration. In this respect the correction method does not improve the more commonly used competitive PCR assay (Freeman et al 1999).

In contrast to the assay using SYBR Green and a TaqMan probe, the assay based entirely on TaqMan probes showed good accuracy and precision when correcting for PCR inhibition by EDTA. The best results were obtained with a house-made master mix based on Bioline Taq polymerase. Inhibition by EDTA reduced the estimated DNA concentration in uncorrected reactions by up to 70% (Fig. 6.10), while the estimates for corrected reactions were unchanged (0.1-0.4 mM) or slightly reduced (up to 6% for 0.6-0.8 mM). The SD of concentrations estimated

using ISO correction was moderately higher than the SD from uncorrected reactions, but any value within the SD error bars was still more accurate than the ones obtained from uncorrected reactions (Fig. 6.9 and 6.10).

6.5.3 Correction of other inhibitors

Having optimized and successfully validated the correction method with EDTA, several other inhibitors commonly found in DNA samples extracted from whole blood were tested. These substances had an inhibitory effect on genomic DNA in all three master mixes (Fig. 6.11 and 6.12), but surprisingly the correction did not work for any of these inhibitors. A look at the inhibition curves (Ct against concentration of inhibitor, Fig. 6.14) revealed that the amplification of the ISO was largely immune to inhibition by SDS, heparin, urea and $FeCl_3$. Amplification was constant for all inhibitor concentrations in the presence and absence of genomic DNA. Also, replacing the ISO with a plasmid containing the ISO did not change the results.

The present study shows that, at least in the system used here, 1) genomic DNA reacts differently to most tested PCR inhibitors than do oligonucleotides or plasmids and 2) PCR inhibition by EDTA is different from inhibition by the other substances tested.

EDTA is thought to inhibit polymerase by reducing the availability of its co-factor Mg^+. Rossen et al (1992) and Kreader (1996) found that EDTA inhibits PCR in concentrations between 0.1 mM and 1 mM. A similar range was obtained with the Bioline Taq MM. The other two master mixes required more EDTA to archive an effect, probably due to the "self adjusting" magnesium buffer included in the mix. Although it has been reported that EDTA is removed during standard extraction procedures (Khosravinia & Ramesha 2007), subsequent dissolving of DNA in TE can be problematic for low DNA quantity samples where the undiluted extraction is used for PCR. EDTA inhibition is resistant to the commonly used amplification facilitators BSA and gp32 (Kreader 1996), indicating that it acts directly on polymerase through Mg^+ concentration.

SDS has previously been identified as a potential PCR inhibitor (Weyant et al 1990; Brissonnoel et al 1991; Rossen et al 1992). In these studies, inhibitory concentrations ranged around 0.1g/l (0.01%). SDS has been suggested to denature polymerase or inhibit the binding of polymerase to DNA (Weyant et al 1990). Alternatively, SDS could facilitate greater solubility of other inhibitors, that might otherwise aggregate or precipitate (Wilson 1997). Kreader (1996) found that PCR inhibition by SDS was not relieved by BSA or gp32.

Heparin is known to be a complex regulator of various enzymes (Ishii et al 1987). It has been suggested to inhibit topoisomerase through its highly sulphated polyanionic nature, possibly through direct binding (Ishii et al 1987). Similar mechanisms could be acting on the polymerase in a PCR reaction. Inhibition by heparin has been reported above 0.01 U/ml (Abu al-Soud & Radstrom 2001). As heparin is co-purified with DNA in standard extraction methods, it has also been suggested to compete with DNA in PCR reactions (Abu al-Soud & Radstrom 2001). Apart from these effects on polymerase itself, it has been found to have a long-term effect on DNA. Heparinised samples that showed PCR inhibition could be rescued by treatment with heparinase II if they were stored for less than three month (Beutler et al 1990; Satsangi et al 1994). Samples stored for longer than that showed irreversible inhibition, indicating an additional effect of heparin through interaction with DNA itself (Satsangi et al 1994).

Urea has been found to inhibit diagnostic PCR above a concentration of 50mM (Khan et al 1991). A direct correlation was observed between urea level of urine samples and inhibition of PCR in clinical conditions. The authors suggested inhibition of non-covalent bonds by urea, affecting binding of polymerase or primer to DNA (Khan et al 1991). Inhibition by urea has been corrected successfully by alternately binding quenching probe competitive loop-mediated isothermal amplification (ABC-LAMP, Tani et al 2007b).

Iron, in form of $FeCl_3$ has been reported to inhibit PCR at concentrations around 10-25μM (Kreader 1996; Abu al-Soud & Radstrom 2001). It is believed to be one of the main factors making the degradation products of iron-containing proteins like haemoglobin or

lactoferin (Abu al-Soud & Radstrom 2001) inhibitory, although the amount of iron released from degradation of these proteins is not enough to fully explain their inhibitory effects (Kreader 1996). $FeCl_3$ inhibition is also relieved by BSA and gp32 (Kreader 1996).

Most of these studies used a circular bacterial chromosome (Rossen et al 1992; Kreader 1996; Tani et al 2007b) or eukaryotic genomic DNA (Beutler et al 1990; Khan et al 1991; Satsangi et al 1994) as a template to test inhibition, while Weyant et al (1990) used a lambda plasmid. Abu al-Soud & Radstrom (2001) developed a test utilizing an oligonucleotide composed exclusively of adenosine nucleotides with a thymine primer already annealed. They measured the increase of SYBR Green fluorescence during a 30 min incubation step. While this procedure eliminates primer dimer formation and other potentially PCR inhibiting factors independent of polymerase, it is unclear to what extent the results can be applied to a real PCR.

The results obtained in the present study contradict the suggestion that polymerase is the main target of PCR inhibition (Abu al-Soud & Radstrom 1998; Radstrom et al 2004). All inhibitors apart from EDTA showed no or little effect on ISO or plasmid, indicating that the activity of polymerase was not altered. Nuclease digestion of template DNA has been proposed as a polymerase independent pathway of PCR inhibition (Abu al-Soud & Radstrom 2000). This possibility is unlikely in the preset study as all reactions contained the same DNA sample and inhibitors were treated equally (same H_2O for dilution, same plastic ware, etc.). In addition we would expect the ISO to be digested more rapidly than the genomic target DNA if nuclease digestion occurs.

One possible explanation for the selectiveness of inhibitors is the specific interaction of the inhibitor with genomic DNA or components attached to it, as suggested for the long storage effect of heparin (Satsangi et al 1994). This seems unlikely, as DNA extraction for the inhibition assays was highly stringent involving two rounds of phenol/chloroform extraction followed by one round of chloroform and precipitation with ethanol. The resulting DNA strings were transferred to TE to avoid contamination with substance and damaged DNA that would co-

precipitate during the typically used centrifugation step (e.g. Sambrook & Russell 2001). The resulting DNA solution was further purified with a commercial kit (Qiagen DNeasy Blood & Tissue Kit) to assure highest DNA quality. All DNA binding proteins should be separated from the DNA by this procedure. Furthermore, Abu al-Soud & Radstrom (2001) reported inhibition of oligonucleotide amplification by heparin and iron in the concentration range tested in the present study, indicating that DNA complexity or bound proteins are not a general requirement of inhibitor activity.

The factors immunizing ISO and plasmid against inhibition by various substances commonly found in DNA extractions remains unknown. Different susceptibility to inhibition has been found for a variety of polymerases and their buffer systems (Abu al-Soud & Radstrom 1998). Further investigations should show if the observed effect is specific to the system used in the present study or if it is a common feature of PCR.

6.5.4 Reliability of internal standard oligonucleotides

This study shows that PCR amplification can be highly selective. In the system used, the amplification of genomic DNA was inhibited by several substances at their previously reported effective concentrations, while amplification of ISO and plasmid remained unchanged. This phenomenon can have serious consequences. For example in diagnostic PCR if the internal standard shows no sign of inhibition, but the target amplification might be inhibited. The result would be not only a false negative diagnosis, but also a high confidence in this (wrong) result, because the reaction supposedly has been controlled for inhibition.

An equal inhibition of target and internal standard should not be taken for granted and should be validated for each assay that uses an internal standard. The protocol presented here can be easily added to any existing assay or incorporated into the development of new assays. If target DNA and internal standard do not show an equal inhibition tested with some (or better all)

inhibitors listed in Table 6.2, then the assay should be deemed unreliable and alternative setups must be employed.

Equally, response to different inhibitors should be tested in gene expression studies. Nolan et al (2006b) suggested the use of an oligonucleotide harbouring a fragment of the potato gene phyB to test inhibition in RT-PCR products (SPUD-assay). It assumes an equal inhibition of c-DNA and SPUD-oligonucleotide. This assumption should be tested by subjecting both to known inhibitors to assure the ability of the SPUD-assay to detect inhibition of target DNA.

Given our lack of knowledge of PCR inhibition, any protocol improving the confidence in Q-PCR should be considered. The here presented results might be specific to the system employed. In this case, the correction method demonstrated for EDTA might be a valuable tool for inhibition correction of all inhibitors in other systems. However, if the here described immunity of ISOs to certain inhibitors is reproducible in other systems, an urgent validation of clinical tests using internal controls is necessary. We can not be too careful when dealing with the health of humans and animals. This includes a recently suggested modification of telomere Q-PCR using oligonucleotides to quantify telomere length in absolute terms (O'Callaghan et al 2008), in which amplification efficiency is widely ignored.

6.6 Reference

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7. General discussion

When Harley et al (1990) and Hastie et al (1990) demonstrated a decline of telomere length (TL) with age in somatic cells *in vitro* and *in vivo*, the field of telomere research emerged quickly. For many years TL has mainly been investigated for its role in cancer and ageing (Wright & Shay 2005; Bolzan & Bianchi 2006). Subsequently, the utility of TL as a biomarker for cancer (Bisoffi et al 2006; Meeker 2006), onset of age-related diseases (Klapper et al 2001) and survival rate in elderly (Cawthon et al 2003; Bischoff et al 2006) has been tested.

Telomere length has also been suggested as a tool to age animals in field studies (Haussmann & Vleck 2002). The results obtained with this approach from several bird species were contradictory and correlated closely with research groups. The present study was designed to test whether molecular ageing is useful in kakapo and other New Zealand bird species. A precise method to measure telomere length is necessary to accurately age birds, and potentially other vertebrates, based on their TL. Two methods have been explored in the present study: telomere restriction fragment (TRF) analysis and telomere quantitative PCR (Q-PCR).

7.1 Measurement of telomere length

TRF and Q-PCR have been chosen in the present study because they require less specialized equipment and have sufficient throughput capacity to screen the sample numbers obtained in ecological studies (Section 2.1.6).

7.1.1 Telomere restriction fragment (TRF) analysis

TRF is the most frequently used method to measure telomere length (Baird & Kipling 2004). Despite its widespread use, it is prone to various measurement error sources and requires careful validation (Section 2.2). Of particular concern, the present study found that the most

commonly used form of background correction (minimum background correction) can make data combined from different gels/blots unreliable (Section 2.2.5.1). Comparison of telomere length between publications is also hampered by differences in the calibration of the gels and the analysis formula used. Unfortunately, about a quarter of the 79 TRF publications listed in Appendix Table II.I do not sufficiently describe the experimental procedure and analysis method used, and more than half of the publications do not include any replication in the assays. Given the variety of error sources identified in the present study (Section 2.2), this is clearly unacceptable practice.

For a TRF publication to be rigorous it should:

1) clearly state the experimental setup. In particular, it should provide details of whether in-gel hybridization or Southern blot was used, whether the DNA was denatured and what probe chemistry and detection method was used.
2) clearly state the gel calibration method and analysis formula used to calculate TL.
3) provide a picture of one gel/blot so the reader can assess the quality of the data.
4) have one sample run at least once on each gel/blot to estimate inter-gel variation and at least one sample run several times on one gel to determine intra-gel variation.

Authors, reviewers and editors should be aware of the multiple pitfalls of TRF (Section 2.2) and ensure sufficient quality controls are in place. Correlations between telomere length and age, stress, survival or susceptibility to diseases should always be interpreted with respect to the inter- and inter-gel variations of the specific assay.

7.1.2 Telomere quantitative PCR (Q-PCR)

It is easy and tempting to take real-time PCR results generated by analysis software for granted. This is especially true because its high throughput capacity makes real-time PCR prone to non-random error sources like amplification efficiency. It is easy to include duplicates, triplicates or even more replication of the same sample in the assay, as PCR reactions are cheap

and fast. However, as shown in the present study (Section 5.3) obtaining the same quantification results from several independent replicates is not a guarantee for accurate results in Q-PCR. A common guideline of real-time PCR is to optimize the reactions to an amplification efficiency of around 100% for the standard curve (e.g. Nolan et al 2006), but even this minimum requirement is ignored by many researchers (e.g. Zhang et al 2007). However, the optimization of the standard curve efficiency alone is not enough to assure reliable results if the efficiency of the samples differ from the standard curve efficiency (Chapter 5). Therefore, the efficiency of the samples should always be tested.

The example of kakapo telomere Q-PCR showed that replication alone does not ensure accurate results (Chapter 5). The mean coefficient of variation (9.8%) was slightly higher than that reported by Cawthon (2002, 5.8%) and Callicott & Womack (2006, 7.1%), but still within an acceptable range. The lower mean CV in the later studies is probably due to measurement of CVs within PCR runs, whereas in the present study CVs were measured in different PCR runs on different days. Nevertheless, the TL of kakapo measured by Q-PCR did not correlate with the TL for the same samples measured by TRF. Analysis of the efficiency of a subset of samples revealed that the measurement error within different dilutions of the same sample was strongly correlated with the amplification efficiency difference between the standard curve and the sample. The more the sample efficiency differed from the standard curve efficiency, the more measurement error occurred (Section 5.3).

The influence of efficiency error is magnified when small differences between individuals are measured, as is the case in studies of the correlation between age-independent telomere length and factors like survival rate or susceptibility to age-related disease (e.g. Cawthon et al 2003; Brouilette et al 2007). Also, the choice of single copy gene should be made more carefully and primers have to be validated *in-silico* and *in-vitro* before used for telomere Q-PCR (Section 5.4.3). A normalization of telomere Q-PCR to a gene with variable copy number

between individuals or single copy gene primers that amplify additional loci that might vary between individuals can compromise any reliability of this method.

7.1.3 Reliability of telomere length measurement

Both methods (TRF and telomere Q-PCR) explored in the present study are prone to substantial measurement errors if not optimized and validated properly. Unfortunately, the need for rigorous quality control of telomere length estimates is not commonly known or if known, is perhaps deliberately ignored. Many studies rely on commercially available kits (e.g. Telo TTAGGG Telomere length assay (Roche) or Telomere Length and TRAP Assay Kit (BD Biosciences)), which may convey a false feeling of security and confidence in the results. Southern blot based TRF assays, and especially the non-radioactive ones used in the mentioned kits are complicated procedures that require a fair amount of experience and optimization to obtain high quality results. Even this level of experimental precautions are missing in many studies utilizing kits to measure telomere length, with very questionable results in some studies (e.g. Binz et al 2005; Bonab et al 2006; Hsu et al 2006).

In general, studies that measure TL as just one of several biological factors, for example when cloning animals (Lanza et al 2000; Gupta et al 2007; Jang et al 2008) or investigating longevity (Jennings et al 1999), tend to show less reliability of results judged from gel/blot pictures if shown. For many of these studies, no gel/blot picture is presented, perhaps because authors do not think they are necessary or because they are forced to omit them due to space constrains imposed by the journal. As such multifunctional analyses often provide new directions and interest in a the research field, it is worrying that even these studies are usually missing quality controls for their telomere length measurement.

Even more concerning is the neglect of some fundamental principles of real-time PCR and general standard curve analysis in some telomere Q-PCR publications. For example in Zhang et al (2007), the amplification efficiency for the telomere reaction is 330%, which is far

beyond any reliable range of real-time PCR. In addition, in this study half of the sample Cts are outside of the range of the standard curve. In this case, Zhang et al (2007) presented a picture of their standard curves that enabled the identification of error sources (Horn T 2008). In contrast, many authors do not (see Appendix Table II.II).

Although results from both methods, TRF and Q-PCR, have been found to correlate with each other, a coefficient of determination (R^2) between 0.65 and 0.82 (Appendix Table II.II) leaves room for interpretation what causes the remaining 20% to 35% ($1-R^2$) difference in TL not explained by the correlation. They might be random errors of the different methods, but might as well be systematic differences between the methods yet to be identified. Analysis of TL inheritance in humans showed signs of X-linked inheritance using TRF (Nawrot et al 2004) while studies using Q-PCR concluded paternal inheritance (Nordfjall et al 2005; Njajou et al 2007). It would be interesting to measure the samples from Nawrot et al (2004) using Q-PCR.

Telomere length as a marker for age and fitness in animals is just a small part of the field of telomere biology. The main focus of telomere length measurement was, and still is, on clinical applications to cancer research. Many of the examples of unreliable telomere length measurements presented here (Section 2.4.4, 5.4.2-3 and Appendix II) were identified in cancer-related publications. From an ethics perspective, stringent quality control should be implemented when TL is used as a prognostic marker of cancer or to assess the success of traditional and new treatment methods for cancer, because misdiagnosis can lead to have life and death decisions.

7.1.4 Real-time amplification efficiency correction

In the present study, the reliability of real-time PCR results in telomere Q-PCR was found to be compromised by different amplification efficiencies between samples, most likely due to the presence of PCR inhibitors (Chapter 5). Currently, there is no standard method to correct for variation in amplification efficiency and this problem has largely been ignored (Bustin 2000;

Bustin et al 2005). The only exception is in diagnostic PCR, where internal standard oligonucleotides (ISOs) are widely, but not mandatorily (Hoorfar et al 2003), used to identify false negatives (Drosten et al 2000; Stocher et al 2003; Nolte 2004; Burggraf & Olgemoller 2004; Hodgson et al 2007).

In the present study, a new assay, based on ISOs, was developed to correct for differing amplification efficiencies quantitatively, with the goal of improving telomere Q-PCR, but also other quantitative assays (Chapter 6). The new method successfully corrected EDTA inhibition, but failed when tested with other common inhibitors, due to a differential response of target DNA and ISO to the inhibitors. While the cause of this phenomenon is unclear, the consequences would be far-reaching if it occurs in other reaction systems. Internal standards are used frequently in diagnosis of pathogens in clinical samples (Stocher et al 2003; Hodgson et al 2007), donor blood (Drosten et al 2000) and food (Malorny et al 2003). Although all diagnostic PCRs should be validated initially (and preferably frequently) by gold standards, like culture of pathogens on diagnostic media, antibody testing or enzyme-linked immunosorbent assay (ELISA) (Khan et al 1991; Drosten et al 2000; Hoorfar et al 2004), financial and time constraints might limit verification of results in practice. Furthermore, detection of genetic modification vectors or non-cultivatable micro organisms can not be validated by culture assays. In this cases diagnostic real-time PCR relies solely on the reliability of the internal standard oligonucleotides. Failure to detect pathogens in clinical samples, donor blood or food can have serious consequences for individuals or even large groups of people.

The present study shows that, in the system used here, ISOs are not able to detect inhibition of target DNA caused by common inhibitors. Consequently, diagnostic PCR assays using ISO's should be urgently investigated for their ability to detect inhibition of their target DNA amplification. Following the experimental setup presented in Section 6.4.2, a simple test would be to add different concentrations of the most frequently expected inhibitors to the reactions. The results would either identify potentially dangerous error sources of diagnostic

PCR or improve the confidence in these assays if amplification of ISO is equally inhibited to target amplification in these assays. The proposed testing is relatively simple to perform, but would be extremely beneficial regardless of the outcome.

7.1.4 Future directions

Although the present study explored many variables associated with telomere length measurement, several factors could not be examined. One of them is the difference between TRF using radioactive in-gel hybridization and a chemiluminescent Southern blot. Only the Southern blot method was performed in the present study. The main difference between these methods is the denaturing of DNA necessary for a Southern blot, which allows binding of telomere probe to the whole fragment as opposed to binding only to the G-rich 3' overhang with the radioactive method. It is unclear if this methodological difference would lead to a different signal distribution within each smear, but it would be interesting to compare results obtained by both methods for the same samples.

The telomere lengths of some bird species (kakapo, Buller's albatross and NZ robin) in the present study were close to the upper resolution limit of Southern blot TRF using constant field electrophoresis. Although still motile, the resolution of the largest fragments was limited, leading to higher measurement errors in species with longer telomeres (e.g. compare error of European sea bass and kakapo, section 2.2.4.4). Pulse field electrophoresis could increase the resolution of the gel and reduce the measurement error for species with longer mean telomere length.

There are a variety of inhibitor-specific and general PCR facilitators shown to counteract PCR inhibition (reviewed in Wilson 1997; Radstrom et al 2004). Although the nature of potential inhibitors present in the kakapo samples is not known, it might be worth investigating whether any of the PCR facilitators identified (e.g. BSA, gp32 (Kreader 1996) or DMSO, PEG 400 (Radstrom et al 2004)) can relieve inhibition in these sample.

Finally, as suggested above, the response of ISO's to inhibitors should be tested in all systems using internal standards. If other systems do not show a differential response of target DNA and ISO, then the method presented for EDTA (Section 6.4.1) might be very useful to quantitatively correct for inhibition in these systems and should be further explored.

7.2 Molecular ageing (MA)

7.2.1 Benefits of molecular ageing

Age information is a missing factor in most field studies of unmonitored populations (Haussmann & Vleck 2002). In birds, where few to no morphological markers for age exist, longitudinal studies are the only possibility to obtain reliable age data (Nisbet 2001). Although there are some long term projects with age data on birds spanning a significant time span, they are rare due to limited long term funding and limited working life span of researchers (reviewed in Nisbet 2001). Molecular ageing (MA) has been suggested as a way to overcome these limitations and to provide detailed knowledge about the age structure of a population if a small subset of individuals with known age are available for calibration (Haussmann & Vleck 2002; Haussmann et al 2003a; Haussmann et al 2003b). In this way, MA could be very useful to test assumptions about the relationships between age and ecological traits like, for example, the increased reproductive success up to peak reproductive age and possible senescence late in life found in some birds (Moller & De Lope 1999; Nisbet 2001; Catry et al 2006).

A simple ageing tool would also have wide applications for wildlife management and species conservation, for example assisting the recovery of the critically endangered kakapo. Although the kakapo population is heavily managed, the recovery programme relies on natural breeding seasons, which are highly irregular (2-7 years, Eason et al 2006), and the assumption of ongoing reproduction efforts by all birds. Recently, however, the oldest kakapo, Richard Henry, showed signs of reproductive senescence (see Chapter 3), similar to findings in other bird species

(Moller & De Lope 1999; Catry et al 2006). MA could provide information on the onset age of senescence in kakapo, which could be used to identify individuals about to leave the reproductive pool. Such knowledge would alter kakapo management. For example, if we knew that a large part of the population is close to reproductive senescence, greater emphasis could be placed on getting these individuals to contribute to the next generation (e.g. via artificial insemination or cryopreservation of sperm for future artificial inseminations (reviewed in Saint Jalme 2002)). Birds suitable for these intensive procedures could be chosen based on heterozygosity and remaining reproductive lifespan. In other endangered bird species, individuals translocated between populations for genetic rescue (Jamieson et al 2008) could be chosen to maximize their reproductive life span in the new population.

Molecular ageing could also help to clarify the endangered status of threatened species. Wrong assumptions about the age structure of a population can lead to disastrous consequences for a species if human intervention, or non-intervention, is necessary. The underestimation of mean age of a birds population might lead to an overestimation of reproductive frequency, reproductive success and viability of the population. Birds that were thought to be reproductively active might already have left the reproductive pool and conservation efforts might start too late.

Inaccurate age assumptions have driven a number of fish species to endangered status (reviewed in Campana 2001). In these cases, a too low age estimate for individuals in the population resulted in an overestimation of reproductive frequency and a too high fishing quota for these species. For example, the orange roughy (*Hoplostethus atlanticus*) has been fished intensively around New Zealand on the basis of a presumed longevity of 20-30 years (van den Broek 1983). Later studies revealed a maximum life span of over 100 years and extremely slow growth rate (Smith et al 1995). At this point the population had already been fished almost to the point of population collapse (Campana 2001). Several other fish species have been overfished based on inaccurate age estimation, including rockfish (sebastes spp) and walleye pollock (*Theragra chalcogramma*) (reviewed in Campana 2001).

7.2.2 Studies of molecular ageing

In 2002, Haussmann & Vleck (2002) found a correlation between telomere length and age in zebra finch (*Taeniopygia guttata*). Haussmann et al (2003b) also reported correlations in tree swallow (*Tachycineta bicolor*), Adelie penguin (*Pygoscelis adeliae*), common tern (*Sterna hirundo*) and Leach's storm-petrel (*Oceanodroma leucorhoa*). In contrast to these findings, Hall et al (2004) and Pauliny et al (2006) reported only weak or no correlation between TL and age in adult European shag (*Phalacrocorax aristotelis*), wandering albatross (*Diomedea exulans*), sand martin (*Riparia riparia*) and dunlin (*Calidris alpine*). In 2008, Haussmann & Mauck (2008a) revealed a possible reason for the difference between their results and the results of other research groups: the telomere optimal estimate (TOE) method (see Section 2.2.5.5).

The research of Haussmann and colleagues has largely driven the development of molecular ageing in birds. Unfortunately, the methodologies used to calculated TL in various Haussmann publications (e.g. Haussmann & Vleck 2002; Haussmann et al 2003a; Haussmann et al 2003b) have not been clearly defined. For example, no information is given on the analysis window of the five bird species in Haussmann et al (2003b). The analysis window in Haussmann & Vleck (2002) has been reported to be 3 kb - 17 kb although later publications describe this analysis as using the whole smear (Haussmann & Mauck 2008a). These inconsistencies make the use of Haussmann's proposed molecular ageing methodologies problematic. The TOE method for molecular ageing (Haussmann & Mauck 2008a) was introduced to remove subjectivity from telomere length analysis that has been introduced by the authors themselves in earlier publications. Furthermore, the TOE method relies on a decline of TL with age in birds, an assumption only supported by Haussmann and colleagues earlier studies with unpublished methodology. It is based on a circular argument such that it is optimized on the premise that all earlier methodologies of Haussmann and colleagues are correct in identifying a correlation between TL and age.

The main hurdle to molecular ageing is the high variation between age-matched individuals (age-independent TL) that has been reported by all groups (Haussmann et al 2003b; Hall et al 2004; Pauliny et al 2006; Juola et al 2006; present study). In fact, this variation seems to be a conserved feature in the animal kingdom as it has been reported in cattle (*Bos taurus*) (Miyashita et al 2002; Ortegon et al 2007), baboon (*Papio spec.*) (Baerlocher et al 2007), dog (*Canis lupus familiaris*) (McKevitt et al 2002), donkey (*Equus asinus*) (Argyle et al 2003), goat (*Capra aegagrus hircus*) (Betts et al 2005) and human (*Homo sapiens*) (e.g. Nawrot et al 2004; Baird et al 2006).

Age-independent TL is influenced by two factors: TL in the zygote and telomere rate of change (Baird 2006). Substantial differences in TL have been found in human sperm (Baird et al 2006), but it is not clear how TL is regulated in the zygote. Telomere length of cloned animals can apparently be readjusted to be equal (Betts et al 2001; Miyashita et al 2002) or higher (Lanza et al 2000; Jeon et al 2005) than those of non-cloned age-matched animals, but the mechanisms of readjustment are unknown.

The telomere rate of change (TROC) has been associated with a variety of factors in human, including stress, diet, and the activity of the immune system (see below). In the European shag, 61% of the variation in TROC between individuals could be explained by three factors: TL as a chick, mass in relation to skeletal size and seasonal effects. In addition, negative selection against birds with shorter telomeres has been suggested as the reason for a lack of birds with short telomeres in cross-sectional studies in Leach's storm-petrel (Haussmann & Mauck 2008b). Such examples suggest that TL might be influenced by environmental factors. While the difference in TL between age matched individuals compromises the precision of molecular ageing, it might be useful for predicting reproductive success and survival rate in birds (Haussmann et al 2005; Pauliny et al 2006, see below). TL might be an indicator of these parameters, but it cannot simultaneous predict age and fitness, as suggested by Haussmann & Mauck (2008a), because these parameters are widely exclusive. For example, a change of TROC

in response to body mass or birth date in the season, as reported by Hall et al (2004) will inevitably distort any correlation between TL and age.

In adult kakapo (Chapter 3) and kea (Section 4.1.1), there were no signs of TL change with age, which is comparable to results from Hall et al (2004) and Pauliny et al (2006). Although TL declined between chicks and old adults in New Zealand robin and saddleback, high age-independent variations and small differences between chick and adult TL made molecular ageing impossible in these species. The only species where MA might be successful was Buller's albatross, which showed high differences between chicks and adults of 20 years age. However, comparing the data obtained from Buller's albatross with the only study that actually applied molecular ageing to assess the age structure of a population (Juola et al 2006), it is unlikely that MA would be able to accurately age individual Buller's albatrosses.

7.2.3 Future of molecular ageing

The present study supports the results of Hall et al (2004), Pauliny et al (2006) and Juola et al (2006) that molecular ageing is not feasible in birds due to extensive age-independent TL differences and a lack of TL decline in adult birds. There seems to be a decline of TL between chicks and adults in most birds, but it has little practical use for ageing as chicks are obviously distinguishable from adults in the field.

The recently suggested TOE method (Haussmann & Mauck 2008a) did not improve MA estimates for kakapo, NZ robin or saddleback. Differences in the experimental setup (e.g. DNA concentration or chemiluminescent detection) between the present study and that of Haussmann & Mauck (2008a) might be responsible for this failure and an exact reproduction of the suggested protocol might have shown some positive effect. However, the only documented benefit of the TOE method is an improvement of the coefficient of determination (R^2) (Haussmann & Mauck 2008a). This alone is not enough to enable accurate age estimation based on TL the bird species investigated in the present study or any other published study. For

example, the study of Juola et al (2006) showed that even an R^2 of 0.82 is not sufficient to accurately age birds. Unfortunately, molecular ageing in its current form does not work and should not be promoted against better knowledge (Haussmann was co-author of the Juola et al study).

The current difficulties of MA appear to arise from biological and not from experimental constraints. Although precise measurement of TL is difficult, the limiting factor is still the high variability of TL between age-matched individuals. ME was not successful in birds using erythrocytes in the present and other studies (e.g. Hall et al 2004; Juola et al 2006), but there are more cells available from low-invasive sampling. Staying with blood, there are a variety of white blood cells available. Unfortunately, they are likely to reflect environmental influences even more than erythrocytes, as they are involved in stress response, mainly through the immune system (Goronzy et al 2006).

For birds, feathers provide an alternative source of DNA. Feather follicles contain three different cell types (Yue et al 2005), whose telomere length might collectively or separately change with age, possibly through regular moulting of the birds. This approach can not utilize TRF and Q-PCR, as the DNA yield from feather extraction is very low. In theory, the single telomere length assay (STELA, Baird et al 2003) should be able to measure the TL of single chromosomes from very low DNA quantities. The subtelomeric binding sites required for this method are not yet available for birds, but might become obtainable as sequencing technology improves rapidly and the benefits of molecular ageing might justify the effort required to obtain this information.

7.3 Other applications of telomere length

While compromising accurate age estimation, age-independent telomere length has been suggested as a marker for stress, fitness and survival. Studies in human showed that TL can be linked to survival rate (Cawthon et al 2003), stress (Epel et al 2004) and onset of age-related

diseases (Fitzpatrick et al 2007). In birds, age-independent TL has been associated with survival rate (Haussmann et al 2005) and reproductive success (Pauliny et al 2006).

7.3.1 Application of age-independent telomere length

In wildlife management and species conservation, a marker for individual fitness would be even more valuable than a marker for age (Pauliny et al 2006). A correlation of TL and reproductive success would allow better choice of birds for breeding programmes and translocations between populations based on TL. While molecular ageing could have been a tool to identify birds that should be supported to pass their genetic makeup to the next generation before they are lost from the gene pool, prediction of reproductive success could help choose birds to quickly increase population size. A combination of heterozygosity screening and TL based reproductive success prediction could aid both, rapid population growth and the conservation of genetic diversity (see Section 4.2).

The findings from European sea bass suggest that TL might also be a marker of the stress experienced during an organisms life span (Horn et al 2008, Appendix III). In this study, TL was not correlated with age, but fish showed high variations between age groups, and low variations within groups. Telomere length was suggested to be linked to different rearing and feeding regimes between the age classes, because they were stocked separately. In this case, TL could be used to improve aquaculture productivity by testing different rearing conditions in respect to TL. In ecology, TL as a marker for stress could be used to investigate effects of environmental stress like habitat fragmentation, global warming or pollution on natural fish populations. Further investigations are necessary, as currently no published results are available for telomere length in correlation to fitness in fish.

There are plans to test the correlation between TL and stress in captive northern bluefin tuna (C. R. Bridges, personal communication). Stress is believed to be the main reason for the

low success of breeding in captive northern bluefin tuna and a marker for stress would be useful for the choice of breeding stock and individuals for hormone-induced spawning.

7.3.2 Studies of age-independent telomere length

Most research on age-independent telomere length has been done in clinical studies on humans. Age-independent TL of leucocytes has been associated with oxidative damage (e.g.Von Zglinicki 2002), stress (e.g. Epel et al 2004), smoking (e.g. Valdes et al 2005), diet and growth (e.g. Demerath et al 2004), exercise (e.g. Cherkas et al 2006), blood pressure (e.g. Demissie et al 2006) and has also been suggested to be a predictor of susceptibility to age related diseases (e.g. Fitzpatrick et al 2007). A comprehensive coverage of factors and mechanisms believed to influence TL and TROC, and their value as a prognostic marker for diseases in humans would go far beyond the scope of this discussion and the reader might be referred to some relevant reviews like Demerath et al (2004), Aviv (2006), Goronzy et al (2006), Rando (2006), and Baird (2006; 2008).

TL regulation seems to be highly complex in humans and the effect sizes of single factors are generally low. For example, in a study by Cherkas et al (2006) smoking, body mass index and exercise were all significantly correlated with TL, but all together accounted for only 14% of the TL differences. Correlations and predictions in humans are difficult, as natural selection is counteracted by social and medical care. Therefore, animals might show more tight correlations between TL and both past stress and future fitness parameters.

Hall et al (2004) found that the telomere rate of loss was strongly (61%) dependent on TL as a chick, mass in relation to skeletal size and seasonal effects in European shag. These early environmental influences seem to be translated into future fitness parameters as TL has been shown to correlate with survival rate in tree swallows (Haussmann et al 2005) and sand martins (Pauliny et al 2006). TL was also correlated with reproductive success in sand martins (Pauliny et al 2006). However, all these correlations are based on small sample sizes, are in the range of

measurement error (Pauliny et al 2006), or were obtained with ambiguous methodology (Haussmann et al 2005) and show low effect sizes (see Section 4.2).

With regard to the present study, kakapo is not a good species to test the effects of TL on reproductive success or survival rate due to the intense management of this species (see Chapter 3). Consequently, no reliable results could be obtained for kakapo TL in relation to stress and fitness.

7.3.3 Future directions

Age-independent TL could become a valuable marker for experienced stress and future fitness. Data on animals other than humans are rare and weak. Low effect sizes might be due to the used of erythrocytes in birds, hence leukocytes might be a better choice as they are directly involved in stress response (Demissie et al 2006).

Unfortunately, the relationship between TL and experienced stress is, once again, blurred by TL variations within age classes. A correlation between stress and TROC, as observed by Hall et al (Hall et al 2004), can only be detected by longitudinal studies. This problem might be less pronounced in other animal taxa. Telomere length in sea bass showed less variation between age classes (Horn et al 2008, Appendix III). Fish might therefore be a good model organism for investigations on stress and TL, as different treatment groups are already available or can easily be generated in aquaculture farms within highly controlled environments. Reproductive success might also be easier to measure in fish than in birds, as fish are external fertilizer and sperm quality can easily be assessed (Abascal et al 2007). However, there is also a need for more data from different taxa (including birds) to investigate if telomere dynamics are conserved among different classes of vertebrates and invertebrates.

7.4 Inheritance of telomere length

Inheritance of telomere length has been found in humans (Baird 2008), mice (Manning et al 2002) and yeast (Askree et al 2004), but has not previously been reported in birds. Mechanisms of TL inheritance are unclear, as contradicting results have been reported (Nawrot et al 2004; Nordfjall et al 2005; Njajou et al 2007, see section 3.4).

In kakapo, 30% of the chick TL could be explained by the TL of the mother. This resembles the paternal inheritance found in humans (Nordfjall et al 2005; Njajou et al 2007), as females are the heterogametic sex in birds. Applications of this finding remain to be explored as they depend on the utility of TL in wildlife management and conservation. Similar to the correlation between TL and reproductive success or survival rate, TL inheritance could aid the choice of individuals for breeding and translocation programmes. In kakapo, inheritance of TL is currently not of practical use for management as TL itself is not a predictor of fitness parameters presently available in this species.

The kakapo results are of great value for general research on TL dynamics, as they suggest that TL inheritance might also be common in birds. Birds have previously been suggested as a model organism to study telomere dynamics and ageing (Holmes & Ottinger 2003), because their TL is more similar to humans than the TL of laboratory mice with their ultra-long telomeres (Kipling & Cooke 1990). Maternal inheritance of TL in kakapo suggests that the telomere dynamics of human and birds are, indeed, very similar and birds might be a good model organism for the exploration of ageing and age related diseases.

7.5 Conclusions

All methods to measure telomere length, and in particular TRF and Q-PCR, are prone to measurement errors and authors, reviewers, editors and readers should be aware of the various pitfalls to ensure reliable results.

Current protocols to measure telomere length in birds do not permit an accurate estimation of age based on telomere length due to high variation between age-matched individuals. A deeper understanding of how these differences arise might lead to the development of telomere length as a marker for experienced stress and ability of individuals to deal with stress in general. Telomere length might then be used to predict fitness parameters like survival rate and reproductive success.

More data on birds and other animals are needed, but the benefits of such markers would be diverse and justify further research on telomere length and fitness parameters in this taxon, as well as other vertebrates and also in invertebrates.

7.6 References

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Appendix I : Protocols

I.I Sampling and Storage

I.I.I Kakapo

Kakapo samples were provided by the New Zealand Department Of Conservation (DOC). Whole blood was snap frozen in liquid nitrogen immediately after sampling and transported to the laboratory. DNA was extracted immediately or after storage at -80°C. Three samples were stored in Queens lysis buffer (Seutin et al 1991) at room temperature for two weeks to test effects of storage conditions on telomere length analysis.

I.I.II Other NZ birds

All other bird samples were stored in Queens lysis buffer (Seutin et al 1991) at room temperature. Buller's albatross samples were provided by Aaron Russ (University of Canterbury, NZ) and Paul Sagar (NIWA) and kea samples by Gyula Gajdon (Universität Wien, Austria). NZ robin and saddleback samples were kindly provided by Dr Ian Jamieson (University of Otago, NZ) and zebra finch samples by Dr. Mark Hauber (University of Auckland, NZ).

I.I.III European sea bass

Data for European sea bass was collected prior to the present study at IFREMER aquaculture station, Palavas-Les-Flots, France. For more details see Appendix III (Telomere length change in European sea bass *Dicentrarchus labrax*)

I.II DNA extraction and quantification

DNA was extracted following standard phenol-chloroform extraction (Sambrook & Russell 2001) with minor changes: ethanol precipitated DNA was transferred to TE (10 mM Tris-HCl, 1 mM EDTA pH 7.5) using a pipette tip. DNA was quantified using Nanodrop ND 1000 (Nanodrop Technology) and associated software.

I.III Telomere restriction fragment (TRF) analysis

I.III.I Optimized protocol

This protocol is base on the 'DIG Application Manual for Filter Hybridization' with some changes. 0.5µg DNA was restricted for approximately 6h at 37°C with 5U *Rsa*I and *Hinf*I (Fermentas Life Sciences) each. Restriction fragments were resolved by constant field electrophoresis in a 0.8% agarose gel for 24h at 50V in TAE (40 mM Tris-HCl, 1 mM EDTA, pH 8). Each gel contained a molecular weight marker (MWM) on the two outer lanes and a minimum of two lanes containing a reference samples for the species examined to estimate the intra- and inter-gel variations. Gels were denatured by soaking in denaturing buffer (0.5 M NaOH, 1.5 M NaCl) twice for 15 min with gentle agitation, rinsed twice with ddH_2O and neutralized twice for 15 min in neutralization buffer (0.5 M Tris-HCl, 1.5 M NaCl, pH 7.5) with gentle agitation. DNA was transferred over night to a positively charged Nylon membrane (Hybond XL, GE Healthcare) by standard capillary transfer using 20xSSC (3 M NaCl, 0.3 M sodium citrate, pH 7). The membrane was cross-linked by UV irradiation (0.120 J/cm²) and prehybridized in a modified Church buffer (0.5 M $NaPO_4$, 1 mM EDTA, pH 7.2, 7% SDS) for approximately 4h at 42°C with gentle agitation while membrane was fixed by four paper clips to assure that it stayed submerged. Hybridization was carried out over night in modified Church buffer with 2.5 µg telomeric probe (digoxygenin-labelled $(TTAGGG)_4$, MWG-Biotech) at 42°C with gentle agitation. The membrane was then washed twice with stringent wash buffer I

(2xSSC, 0.1% SDS) for 5 min at room temperature and twice with stringent wash buffer II (0.2xSSC, 0.1 % SDS) for 20 min at 50°C. The membrane was incubated in blocking buffer (0.1 M Maleic acid, 0.15 M NaCl, pH 7.5, 1% Blocking reagent (Roche)) and after 30 min 7.5 U Anti-Dig-AP Fab Fragment (Roche) were added. Following another 30 min, the membrane was washed twice for 15 min with wash buffer (0.1 M Maleic acid, 0.15 M NaCl, pH 7.5, 0.3% Tween 20) and equilibrated in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9,5) for 5 min. Eight ml of 0.5xCDPStar (Roche, diluted in detection buffer) were applied to the membrane and immediately spread evenly with an overhead transparency sheet. Excess liquid was squeezed out after 5 min incubation and membrane was stored in a film cassette for 1 h prior to signal detection using Hyperfilm ECL (GE Healthcare) with exposure times of typically 10-60 sec.

Films were scanned and the pictures were aligned in Photoshop 7 (Adobe) using the molecular weight markers on either side. Pictures were cropped well below and above any detectable signal, loaded into ImageJ 1.38X (Java 1.6.0_02) and black-white inverted. The picture properties were set to cm and signal distribution for each lane was recorded starting at the bottom of the gel (i.e. against the direction of migration). Signal distributions were copied into Excel (MS), peaks of MWM distribution were identified and the mean of the peaks of both MWM lanes were used to calculate a logistic model fit using CurveExpert 1.38. The resulting formula was used to convert cm migration into molecular weight in kb. The average signal intensity of all sample lanes (excluding MWM) was used as background and subtracted from each point of the signal distributions for all lanes. Mean telomere length was calculated using the formula $\sum(OD_i \cdot MW_i)/\sum OD_i$ with OD being the optical density at the position *i* and MW being the molecular weight at the same position *i*.

I.III.II Alternative conditions used during optimization

Alternative restriction enzymes *Rsa*I, *Dpn*II, *Bfa*I, *Mse*I and *Alu*I were tested to generate restriction fragments. Hybridization was also carried out in the recommended (Roche)

Digoxygenin hybridization buffer (5X SSC, 0.1 % N-lauroylsarcosine, 0.02 % SDS, 1 % Blocking reagent (Roche)).

For image analysis a minimum background was also used, which equalled the minimum signal intensity of all sample lanes. The calibration of the gel was done by exponential fit using CurveExpert 1.38 and Excel (MS). Alternative formulas used to calculate mean telomere length are given in Section 2.2.5.2. The use of the TOE method (Haussmann & Mauck 2008) is described in Section 2.2.5.5.

I.III.III *Bal*31 nuclease digestion

A digestion was prepared to contain 16.45 ng/µl kakapo DNA and 0.03U/µl *Bal*31 (Fermantas). The mix was incubated at 30°C and aliquots of 15.2 µl (250 ng DNA) were taken each 10 min. *Bal*31 in aliquots was heat-deactivated at 80°C for 20 min and aliquots were stored at 4°C for one day before processed according to I.III.I.

I.IV Real-time PCR: Baseline, threshold and efficiency

The following issues apply to MxPro – Mx3000P v3.00 Build 311, Schema 74 software (Stratagene), but might be similar in other platforms.

Although all analysis software are equipped with algorithms to automatically determine baseline and threshold, it is essential to check theses parameters manually (Nolan et al 2006). In reactions with low Ct, the baseline is often misapplied by the analysis programme. Fig. I.I shows an automatically set and a manually corrected baseline.

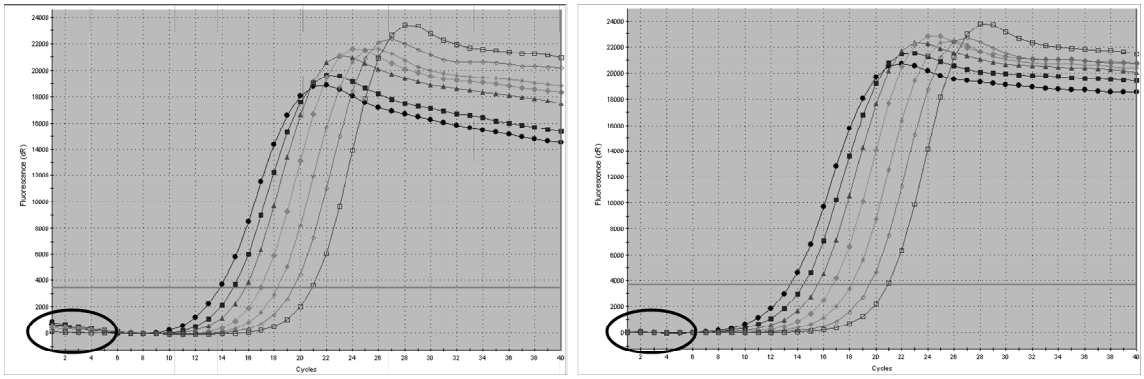

Figure I.I: Manual baseline correction. Baseline estimated by the analysis program (left) and set by hand (right). Note the difference in the first cycles (circle).

The baseline must be checked for each reaction. To estimate the error of DNA quantification arising from wrong baseline setting, a real-time PCR with three standard curves and 72 kakapo samples was performed amplifying the telomeric sequences and signal was detected by SYBR Green (see Chapter 5). Fig. I.II shows the difference between DNA concentration estimated with the automatic baseline setting and the manual correction. Wrong (automatic) baseline setting introduced an error of approximately 2.8% and reduced the efficiency by 3.3%.

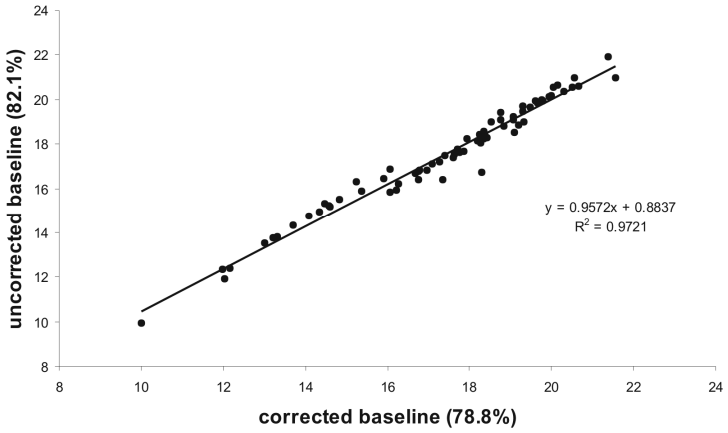

Figure I.II: Effect of baseline setting. Relation between telomeric DNA concentration of 72 kakapo samples estimated with uncorrected (automatic) and corrected (manual) baseline. The amplification efficiency of the standard curve is denoted in brackets.

While these differences are small, they add up with other analysis artefacts like threshold setting. In the same experiment, one reaction, one of the three replicates of the highest

concentration of the standard curve, had a huge influence on the threshold automatically set by the analysis program (Fig. I.III). Although the amplification curve of this reaction did not show any apparent difference from the other reactions (Fig. I.IV), its removal from the analysis resulted in a reduction of the threshold level from 6092 fluorescent units (FU) to 1511 FU (Fig. I.III). The cause of this change is not known and Stratagene customer support assured that it does not have any influence on the measurement as the threshold changes for all samples in the same manner. This statement is not supported by a comparison of estimated DNA concentrations and amplification efficiency using the different thresholds obtained (Fig I.IV).

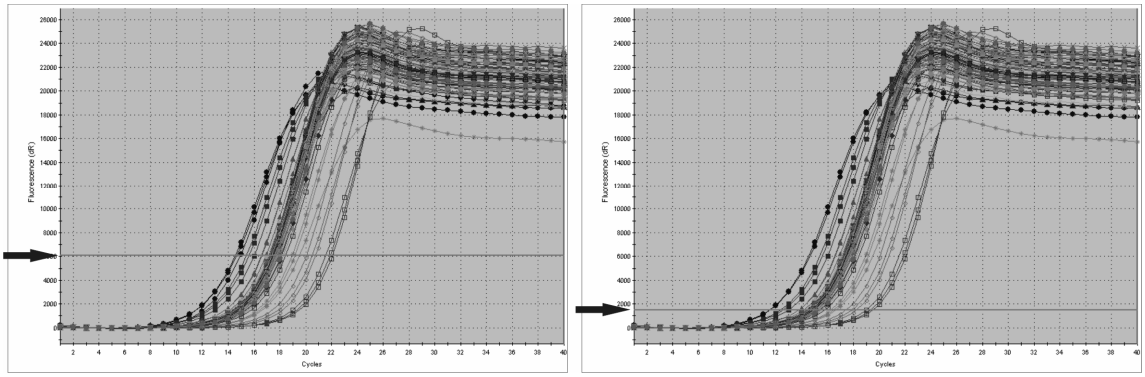

Figure I.III: Drop of threshold due to a single reaction. The elimination of this reaction (shown in Fig I.IV) resulted in a decrease of the automatically set threshold level from 6092 to 1511 FU.

Fig. I.IV shows the change of estimated efficiency and DNA quantity due to the drop in the threshold. The reaction causing the shift (position A12) was the highest concentration in one of the three standard curves in this run. There was no apparent difference between this reaction and its other two replicates (Fig. I.IV left). The elimination of A12 from the analysis resulted in 2.7% measurement difference (1-R^2 between both thresholds) and also in a decrease of estimated amplification efficiency of 8.2% (Fig. I.IV right). To ensure that the change is not due to a change of the standard curve when eliminating one of the triplicates I tested the correlation between the dataset with and without the A12 using a constant threshold (Fig I.IV right inlay). There was no difference between the two datasets under this conditions (R^2=1), indicating that the difference is solely due to the change of threshold level.

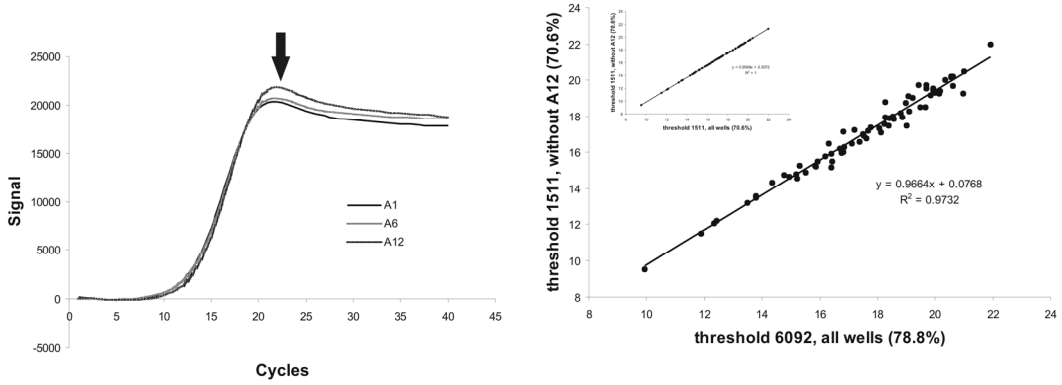

Figure I.IV: Sample causing a drop of threshold and its effects. The sample A12 (arrow) did not show any apparent difference from its two replicates (left), but caused a difference of estimated DNA concentration of 2.7% (1-R^2) and a decrease of estimated efficiency of 8.2% (right). These effects were not due to a change in the standard curve (inlay, see text).

The setting of the threshold is highly subjective (Nolan et al 2006). The main issue, associated with the threshold, is that there is no right or wrong threshold. A wide range can be used for analysis. Unfortunately the estimated amplification efficiency changes widely with the chosen threshold. Consequently, it is much more important to ensure the same estimated amplification efficiency for all samples than desperately trying to get to the recommended values (approximately 90-110%, see chapter 6) for the standard curve. As the efficiency can be changed by moving the threshold up and down, an assay with 80% estimated efficiency in all samples is more reliable that an assay with 100% efficiency of the standard curve, but no estimates for the actual samples.

I.V References

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Appendix II : Tables

II.I Publications using telomere restriction fragment (TRF) analysis

The following abbreviations are used :

Headings of table

hyb. = hybridization (in-gel or southern blot)
probe = label of probe (radioactive, Digoxygenin, Biotin or Alkaline Phosphatase
denat. = denaturing of gel
repli. = replicated measurement of same sample(s)
CV = coefficient of variation or coefficient of determination (R^2) between replicates
R^2 = coefficient of determination (TL against age (years) or population doubling (PD)); taken directly from publications or calculated from r.
loss = loss of telomere length (bp) per year, population doubling (PD, in cell culture) or cm body length
formula = formula used to calculate mean TL :
1 = $\sum(OD_i \bullet MW_i)/\sum OD$
2 = $\sum(OD_i)/\sum(OD_i/MW_i)$
3 = $\sum(OD_i)/\sum(OD_i MW_i)$, but this formula does not produce useful results, maybe a typo ?
ref. = reference

Aberrations used in table

? = information not given or ambiguously explained
CC = cell culture
desc./2xdesc. = as described: The examination of one or two additional papers was necessary to find the missing information
HRP = horseradish peroxidase
intra = intra-gel variation
inter = inter-gel variation
ng. = data must be known, but not given in publication
PBMC = peripheral blood mononuclear cells
UCB = umbilical cord blood
VSMC = vascular smooth muscle cells
WBC = white blood cells

Table II.I: Publications using telomere restriction fragment analysis (TRF).

year	species	tissue	enzymes	hyb.	probe	denat.	picture	repli.	CV	R^2	loss	comments	formula	ref.
1990	human	fibroblast CC	*MspI, RsaI*	blot	radio	?	yes	no	no	no	no	first TRF	2	(1)
1990	human	cancer and adjacent tissue	HinfI or AluI	blot	radio	?	yes	yes	5-10%	ng	33/yr	-	mean of OD ?	(2)
1994	human	lymphocytes CC	HinfI, RsaI	gel	radio	?	yes	no	no	ng	80-120/PD	-	2xdesc. 2	(3)
1994	human	WBC	HaeIII	blot	radio	no	scan	no	no	0.51 yr	31/yr	-	1 ?	(4)
1995	human	endothelial CC + div. tissues	HinfI, RsaI	gel	radio	yes	yes	no	no	0.96 PD	140-190/PD; 87-147/yr	variable correlations for tissues	2xdesc. 2	(5)
1995	mice	fibroblast CC	*HinfI*	gel	radio	yes	yes	no	no	ng	75/PD	-	2Xdesc. 2	(6)
1996	human	fibroblast	desc	desc	desc	desc	yes	no	no	no	no	also Q-FISH	2Xdesc. 2	(7)
1997	human	fibroblast CC	*AluI, CfoI, HaeIII HinfI, MspI, RsaI*	?	?	?	yes	no	no	0.76	49/PD	-	2	(8)
1998	chicken	fibroblast CC	HinfI, AluI	gel	radio	no	yes	no	no	ng	59/PD	-	desc 2	(9)
1998	cow, pig	sperm, kidney	*MspI*	blot	radio	no	yes	3/sample	300bp for all (?)	no	no	-	peak	(10)
1998	human	PBMC	AluI	blot	AP	no	yes	no	no	no	no	-	1	(11)
1998	human	div. tissues	HinfI, RsaI	blot	Dig	yes	yes	no	no	no	no	-	2	(12)
1999	human	CC	*AluI, CfoI, HaeIII, HinfI, MspI, RsaI*	gel	radio	no	yes	no	no	no	no	-	?	(13)
1999	human	PBMC	AluI	blot	AP	no	yes	no	no	no	no		1	(14)
1999	rat	kidney	HinfI, RsaI	blot	radio	no	yes	one control	no	no	no	bad blots	% OD (?)	(15)

Table II.I: Publications using telomere restriction fragment analysis (TRF). (continued)

year	species	tissue	enzymes	hyb.	probe	denat.	picture	repli.	CV	R^2	loss	comments	formula	ref.
2000	chicken	diverse organs	HaeIII	blot	radio	no	yes	no	no	no	no	-	1	(16)
2000	cow	fibroblast	HinfI, RsaI	blot	biotin	yes	yes	no	no	0.54/yr FISH	no	cloned cow, bad blots, also FISH	1	(17)
2000	human	CC	?	?	?	?	no	no	no	ng	49-101/PD	-	?	(18)
2000	human	WBC	HinfI, RsaI	blot	Dig	yes	no	yes	R^2=0.93	not signify-cant	no	twin study, small age range	desc. 2	(19)
2001	human	???	HinfI, RsaI	blot	radio	no	yes	no	no	no	no	telemetric.jar	2	(20)
2001	human	cancer CC	*Hae III, HhaI, HinfI*	blot	radio	no	yes	partly	17% n=4	no	no	correlation withTALA, bad blot	50% total OD	(21)
2001	human	CC	HinfI, RsaI	blot	radio	no	no	no	no	0.45/yr FISH	ng	correlation with Q-	peak	(22)
2002	cynom-olgus monkey	PBMC	AluI	blot	biotin	yes	no	no	no	0.27-0.53	62.7/yr	correlation with Q-FISH	?	(23)
2002	div. dogs	PBMC	HinfI, RsaI	blot	biotin	?	yes	no	no	0-0.23/yr	ng	bad blot	1	(24)
2002	zebra finch	erythrocytes	HaeIII, HinfI, MspI	gel	radio	no	yes	1 sample 6 times	1.3% intra	0.54/yr	516/yr	first molecular ageing of bird	1	(25)
2003	chicken	fibroblast CC	HaeIII	blot	radio	?	yes	no	no	div.	div.	bad blot, TL does not agree with picture !!	1	(26)
2003	div. birds	RBC	*HinfI*	gel	radio	no	no	yes	<1.5 intra and inter	div	div	-	1	(27)
2003	equine	PBMC	HinfI, RsaI	blot	biotin	yes	yes	no	no	ng.	ng.	mostly p-values	1	(28)

Table II.I: Publications using telomere restriction fragment analysis (TRF). (continued)

year	species	tissue	enzymes	hyb.	probe	denat.	picture	repli.	CV	R^2	loss	comments	formula	ref.
2003	human	div. organs	HinfI, RsaI	bot	radio	no	no	no	no	0.58/yr FISH	ng.	correlation with Q-FISH and 0.36 tissue	1	(29)
2003	human	cancer CC	HinfI	blot	radio	yes	yes	no	no	no	no	approximate TL	2	(30)
2003	terns	erythrocytes	*HinfI*	gel	radio	no	no	no	no	0.61	ng.		1	(31)
2004	C. elegans	whole	HinfI	blot	radio	?	yes	no	no	no	no	overexpression of T protein	?	(32)
2004	cow	skin, WBC	desc.	desc.	desc.	desc.	yes	no	no.	no	no	very bad blot	desc	(33)
2004	E. Shag Wand. Alba-tross	blood	HinfI, RsaI	blot	Dig	yes	no	only blot analysis ?	R^2=0.94 ?	0.35 0.36	300/yr 160/yr	no TL shortening in adults	3 ?	(34)
2004	human	fibroblast CC	HinfI	blot	radio	no	yes	yes	only error bars	no	no	correlation with Q-	?	(35)
2004	human	fibroblasts	HinfI	blot gel	AP radio	yes and no	yes	3/sample	ng	no	no	-	1 ?	(36)
2004	Human	PBMC Saliva	AluI, RsaI	blot	Dig	yes	yes	no	no	0.22-0.26	ng.	-	peak	(37)
2004	pig	div. tissue	HinfI, Sau3AI	blot	?	?	yes	no	no	no	no	-	only ranges	(38)
2005	goat	skin, PBMC	HinfI, RsaI	blot	Dig	yes	yes	no	no	0.04-0.62	400-1200/yr	cloned goat	desc. 2	(39)
2005	human	cancer CC	HinfI, RsaI	blot	Dig	yes	yes	no	no	not found	no	-	2	(40)
2005	human	neurobla-stoma CC	HinfI, RsaI	blot	Dig	yes	yes	no	no	no	no	blot very very bad	2	(41)
2005	human	div cancer tissues	desc.	desc.	desc.	desc.	no	no	no	no	no	correlation with Q-PCR	desc.	(42)
2005	human	leucocytes	HinfI, RsaI	blot	Dig	yes	no	2/sample	R^2=0.94	no	no	-	2	(43)

Table II.I: Publications using telomere restriction fragment analysis (TRF). (continued)

year	species	tissue	enzymes	hyb.	probe	denat.	picture	repli.	CV	R^2	loss	comments	formula	ref.
2005	human	WBC	desc.	blot	desc.	desc.	no	no	no	0.21/yr	27/yr	-	desc.	(44)
2005	human	PBMC	HinfI	gel	radio	yes	no	3	ng.	no	no	2 control DNA on each gel, but no values for that	1 ?	(45)
2005	lago-morphs	CC	*AluI, CfoI, HaeIII,HinfI, MspI, RsaI*	gel	radio	yes	yes	no	no	no	no	-	?	(46)
2005	pig, cow		HinfI, RsaI	blot	Dig	yes	yes	no	no	no	no	TL does not agree with picture !!	mean of smallest and largest signal	(47)
2005	Tree Swal-low	erythrocytes	HaeIII, HindIII, HinfI	gel	radio	no	no	no	no	no	no	-	desc. 1	(48)
2006	Alliga-tor	erythrocytes	HinfI, HaeIII, HindIII	gel	radio	no	yes	one sample 3X each gel	intra 1%, inter 3.6%	0.18	8bp/cm	-	1	(49)
2006	Frigate-bird	erythrocytes	HinfI	gel	radio	no	no	one sample 3 X each gel	intra 4.8%, inter 5.4%	0.74-0.82	varies with age	best fit with square-root transformed age	1	(50)
2006	human	T-cells	HinfI, RsaI	blot	Dig	yes	yes	no	no	no	no	also real time + flow-FISH, but no correlation given	2	(51)
2006	human	sperm	HinfI, RsaI or HphI, MnlI	blot	?	?	yes	no	no	no	no	compared enzyme mix + STELLA	2	(52)

Table II.I: Publications using telomere restriction fragment analysis (TRF). (continued)

year	species	tissue	enzymes	hyb.	probe	denat.	picture	repli.	CV	R^2	loss	comments	formula	ref.
2006	human	hemato-poietic sterm cells	HinfI, RsaI	blot	Dig	yes	yes	no	no	no	44/120days	very bad picture	2	(53)
2006	human	bone marrow lymph note	HinfI, RsaI	blot	radio	yes	no	no	no	no	no	-	peak	(54)
2006	human	lung cancer	HinfI, RsaI	blot	Dig	yes	yes	3 ?	no	no	no	-	2	(55)
2006	human	pancreas	HinfI or RsaI	blot	radio	desc.	yes	2	R^2=0.999 or 0.96 ?		36/yr	blot over-exposed, CV not supported by pictures !	peak ?	(56)
2006	human	leucocytes	HinfI, RsaI	blot	Dig	yes	no	2-3	R^2=0.92% ?	0.06, highly significant !?	16/yr	sample measured twice, if more than 5% apart than 3 times and two <5% apart taken	2 ?	(57)
2006	human	leucocytes	HinfI, RsaI	blot	radio	no	yes	2	%	no	no	-	2	(58)
2006	human	cancer CC	HinfI, RsaI	blot	Dig	yes	yes	no	no	no	no	correlation with (3 points !)	2	(59)
2006	human	PBMC	HinfI, RsaI	blot	Dig	yes	yes	no	no	no	no	-	2	(60)
2006	human	leucocytes	desc	desc	desc	desc	no	2	intra 2%	0.17/yr	22/yr	-	desc.	(61)
2006	human	PBMC	desc	desc	desc	desc	no	no	no	ng	19.8/yr	-	desc.	(62)
2006	human	VSMC VSMC CC	HinfI, RsaI	blot	radio	?	yes	no	no	ng	100/PD	compare cancer adjacent tissue	2	(63)
2006	human	skin	HinfI, RsaI	blot	Dig	no ?	no	some	no	0.01/yr	9-12/yr	discussing results with P>0.05 !	?	(64)
2006	human	leucocytes	HinfI, RsaI	blot	Dig	yes	no	2	1.50 %	0.07	31bp/yr	-	2	(65)

Table II.I: Publications using telomere restriction fragment analysis (TRF). (continued)

year	species	tissue	enzymes	hyb.	probe	denat.	picture	repli.	CV	R^2	loss	comments	formula	ref.
2006	human	UCB, PBMC	HinfI, RsaI	blot	HRP	yes	yes	some 3 times	ca. 1.5%	no	no	-	?	(66)
2006	human	leucocytes	desc.	desc.	desc.	desc.	no	no	no	no	no	-	1	(67)
2006	human	PBMC	HinfI, RsaI	blot	biotin	?	no	no	no	no	no	-	?	(68)
2006	human	fibroblast CC	desc	desc	desc	desc	yes	no	no	no	no	-	desc.	(69)
2006	rat	kidney	HinfI	blot	Dig	yes	yes	2	ng	no	no	-	2	(70)
2006	Sand Martin, Dunlin	Blood	HaeIII	gel	radio	no	yes	some 2 times	4.4%, R^2=0.59	div.	div.	-	2	(71)
2006	sea urchins	div. tissues	HinfI, RsaI	blot	Dig	yes	yes	no	no	no	no	-	2	(72)
2006	sheep	fibroblast CC	2xdesc. *MspI, RsaI*	desc. gel	desc. radio	desc. yes	yes	no	no	no	no	-	desc. telorun ?	(73)
2007	cow	sperm, skin, PBMC	HinfI, RsaI	blot	biotin	yes	yes	no	no	0.01/yr	100b/yr	-	desc. 2	(74)
2007	Gaddi goat	fibroblast	HinfI, RsaI	blot	Dig	yes	yes	no	no	no	no	TL decline coincides with diagonal artifact on blot !	1	(75)
2007	Ginkgo	diverse tissues	TaqI	blot	Dig	?	yes	3	?	no	no	-	2	(76)
2007	human	leucocytes	HinfI, RsaI	blot	Dig	yes	1 lane	some	1.76 %	0.66/yr	ca. 32/yr	correlation with	2 ?	(77)
2008	dog	?	HinfI, RsaI	blot	Dig	yes	yes	?	no	no	no	cloned dog	mean of largest + smallest	(78)
2008	human	leucocytes	HinfI, RsaI	blot	radio	no	no	yes	no	no	no	-	1	(79)

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II.II Publications using telomere Q-PCR

The following abbreviations are used :

?	= information not given or ambiguously explained
desc.	= as described, information given in another paper
36B4	= acidic ribosomal phosphoprotein PO gene
GADPH	= glyceraldehyde-3-phosphate dehydrogenase
ng.	= data must be known, but not given in publication
SCG	= single copy gene
T	= telomere
ref.	= Reference

Footnotes :

† the source of the conversion factor from T/S to bp is not clear; the authors description imply an own regression, but sample size and age range do not match their study, but do match Cawthon (1). Nevertheless the conversion factor does not fit the regression by Cawthon (1).

Regressions between Q-PCR and TRF (see also Table 5.3):

$y=1910.5x+4157$, $R^2=0.677$	(1)
$y=3198.9x+3128$, $R^2=0.818$	(10)
$y=1406.1x+8685$, $R^2=0.664$	(12)
$y=1095.4x+6846$, $R^2=0.766$	(18)
conversion factor 4270 without reference, missing value of y-intercept	(19)
conversion factor 2870 with unclear origin, missing value of y-intercept	(8)

Table II.II: Publications using telomere Q-PCR.

year	primer sequence	standard curve efficiency	single copy gene	reference DNA	correlation	experimental error	comments	ref.
2002	yes	no	36B4	one sample	0.677	5.8%-13.1%	first telomere Q-PCR	(1)
2004	yes	SCG: 96% T: 107.9%	36B4	?	no	0.0545 and 0.0326, unknown units	optimization for Roche LightCycler	(2)
2005	yes	no	GADPH 36B4 -actin	unfixed sample	no	no	effect of tissue fixing agents over time	(3)
2006	yes	assessable from figure	36B4	three different cell lines	no	no	cell lines suggested as standards for Q-PCR, but different slopes indicate different efficiencies !	(4)
2006	yes	no	desc.	desc.	no	no	-	(5)
2007	yes	deducible from figure: SCG: 106.5% T: 330%	36B4	desc.	no	no	efficiency of telomere reaction 330%, ca. half of sample Ct outside of standard curve range	(6)
2007	yes	no	36B4	calibrator sample	no	no	-	(7)
2006	yes	no	36B4	reference ?	no	no	unclear origin of conversion factor	(8)
2004	yes	no	GADPH	Roche TFR kit control DNA	0.73	no	-	(9)
2005	yes	no	-globin	cell line	0.818	no	-	(10)
2005	yes	deducible from suppl. figure: SCG: 106.7% T: 100.5%	2-globin	cell line	no	no	-	(11)
2006	yes	no values, but slopes of standard curves look quite different	36B4	one sample	0.664	7.10%	first study in mice	(12)
2003	desc.	no	desc.	desc.	desc.	no	no T/S values given, just statistical results	(13)
2004	desc.	no	desc.	desc.	no	no	-	(14)
2004	telomere	no	desc.	desc. + cell-lines for bp conversion	no	no	-	(15)

Table II.II: Publications using telomere Q-PCR. (continued)

year	primer sequence	standard curve efficiency	single copy gene	Reference DNA	correlation	experimental error	Comments	Ref..
2005	desc.	no	desc.	desc. + three known samples	no		longitudinal study, one individual gained ca. 5kb in 3.7 years	(16)
2006	desc.	no	desc.	desc.	no	no	whole method part desc.	(17)
2006	desc.	no	desc.	desc.	0.77	no		(18)
2007	desc.	no	desc.	desc.	no	samples with intraassay variability >15% excluded	-	(19)
2006	modification of desc. ?	no	36B4	reference ?	ng.	no	no concentrations for PCR reaction components	(20)
2007	desc.	no	desc.	desc.	no	no	-	(21)

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Appendix III : Publications

Letter to the editor : Cancer Investigation, in press

Comments on quantitative real-time PCR for measurement of telomere length

Dear Editor,

I write to you because I am concerned about an article recently published in Cancer Investigation (Zhang, Y., et al. "Telomere length in hepatocellular carcinoma and paired adjacent non-tumor tissues by quantitative PCR." Cancer Investigation 2007, 25, (8), 668-677).

In this article the authors use quantitative real time PCR (qPCR) to measure telomere length in hepatocellular carcinoma and adjacent tissues. I would like to point out two issues:

1) One of the most important variables in every real time assay is amplification efficiency (E) [1;2]. An efficiency of 100% indicates a doubling of DNA in each cycle. Efficiencies between 90% and 110% are generally accepted. Although the authors do not mention the efficiency of their reactions, it can be calculated from the slope values of their standard curves using the equation $E = 10^{(-1/slope)}$ where a value of 2 equals 100% efficiency [2]. Two different qPCR reactions are done to estimate relative telomere length: one for telomeres and one for a single copy gene. For the single copy gene the efficiency is 106.5% and therefore within an acceptable range. In contrast, the efficiency of the telomere reaction is 330.1%. No reliable quantification can be made in this range.

2) Approximately 50% of all sample Ct values for both reactions (single copy gene and telomeres) are outside of the range of the standard curve. A standard curve assay in real time PCR, and in general, is only reliable within the range of the data points represented in the standard curve.

Both issues are especially important for telomere qPCR. Since a ratio of telomere Ct and single copy gene Ct is calculated [3], the error gets amplified in the data analysis.

I think it is especially concerning to see these fundamental mistakes in an article about cancer research. If other researchers adopt this method uncritically it can lead to serious damage when used for risk assessment of patients or the measurement of therapy success.

Sincerely Yours,

Thorsten Horn

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Full research paper : Australian Journal of Zoology 2008, 56, 207-210

Telomere length change in European sea bass (Dicentrarchus labrax)

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Telomere length change in European sea bass (*Dicentrarchus labrax*)

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Abstract. Telomeres, the repetitive sequences found at the end of chromosomes, are observed to shorten with age in birds and mammals, but to date no investigation on changes of telomere length has been made in long-lived marine fish during ageing. We have measured the telomere length of European sea bass (*Dicentrarchus labrax*) ranging in age from 12 to 94 months. No overall decrease of telomere length could be detected, but a broad range of intraspecies variation was observed. Telomere length change does not appear to be useful for estimating age in this species, but may prove a useful tool for examining individual fitness and response to stress.

Introduction

Telomeres are located at the ends of chromosomes and consist of a repetitive DNA sequence (TTAGGG in vertebrates: Harley and Villeponteau 1995; Zakian 1995) and associated proteins (Bertuch and Lundblad 2006). Their functions include prevention of chromosomal degradation and fusion (Bailey and Murnane 2006), regulation of subtelomeric gene expression (Harley and Villeponteau 1995) and positioning of chromosomes during cell division (Kirk *et al.* 1997). Telomeres also buffer against loss of terminal DNA during cell replication, as the length of telomeres decreases with each cell division, owing to the inability of the DNA polymerase to replicate the end of chromosomes (Watson 1972). This shortening is believed to act as a protection mechanism against cancer, a disease characterised by uncontrolled cell division. If telomere length falls below a certain threshold cells enter senescence or apoptosis (Ju and Rudolph 2006), inhibiting further replication. Therefore, telomere shortening limits the replicative capacity of somatic cells to a finite number of cell divisions (Klapper *et al.* 2001). In mammals, the only exceptions to this rule are stem cells and gonadal tissues. In these cells telomere length is maintained by telomerase, an enzyme that is capable of adding new telomeric sequence to the end of the chromosome (Klapper *et al.* 2001). Telomerase activity has been found in most tissues of rainbow trout (*Oncorhynchus mykiss*) (Klapper *et al.* 1998) and extracts of whole rainbow trout embryos (Yoda *et al.* 2002).

The shortening of telomeres in tissues such as nucleated red blood cells has been proposed to be useful for ageing whole animals (Haussmann and Vleck 2002) and this approach has been applied to different bird species with varying degrees of success (Haussmann and Vleck 2002; Haussmann *et al.* 2003*a*, 2003*b*; Hall *et al.* 2004; Juola *et al.* 2006; Pauliny *et al.* 2006). In fish, which also possess nucleated red blood cells, such a non-lethal ageing technique (Nakagawa *et al.* 2004) could supplement or replace present methods and would be vital for fishery and species conservation methods. Productivity of a fish population is highly dependent on its age structure and inaccurate age estimations have driven several fish species to endangered status (reviewed in Campana 2001).

Recently, a decline of telomere length with age was noted in the short-lived freshwater medaka (*Oryzias latipes*) (Hatakeyama *et al.* 2008). However, tissues used in that study required lethal sampling.

Here we examine the change in telomere length in red blood cells with age in a commercially important marine aquaculture fish species, the European sea bass (*Dicentrarchus labrax*), providing the first test of the utility of telomere ageing in aquaculture and also as a fisheries-management tool.

Materials and methods

All specimens of sea bass of known age were obtained from different fish stocks reared in the IFREMER-Station, Palavas-Les-Flots, France. The study fish were the result of aquaculture breeding of wild-caught sea bass from the Mediterranean. The ages of cross-sectional sampled fish ranged from 12 to 94 months (Fig. 1). Blood was collected in Heparin-rinsed syringes from the heart or caudal veins and DNA was extracted from whole blood using a QIAamp DNA Blood Mini Kit. Telomere length was measured using the Telomere Restriction Fragment Assay (Harley *et al.* 1990), which has been shown to measure telomere length in fish (Hatakeyama *et al.* 2008). Genomic DNA (0.75 μg) was cut with *Rsa*I and *Hin*fI and resolved in a 0.8% agarose-gel in TAE buffer for 6 h at 100 V. The gel was denatured and DNA transferred to a positively charged nylon membrane by capillary transfer. Hybridisation was carried out at 42°C using a Digoxygenin-labelled telomere probe $(CCCTAA)_4$, and signal

10.1071/ZO08046 0004-959X/08/030207

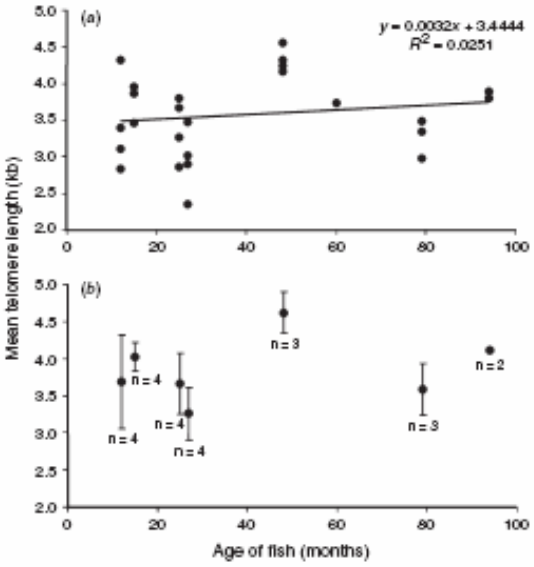

Fig. 1. Telomere length measurement of the European sea bass (*Dicentrarchus labrax*). (*a*) Telomere length of all individuals measured ($n = 24$). (*b*) Mean telomere length of each age-class (s.d. is indicated by error bars).

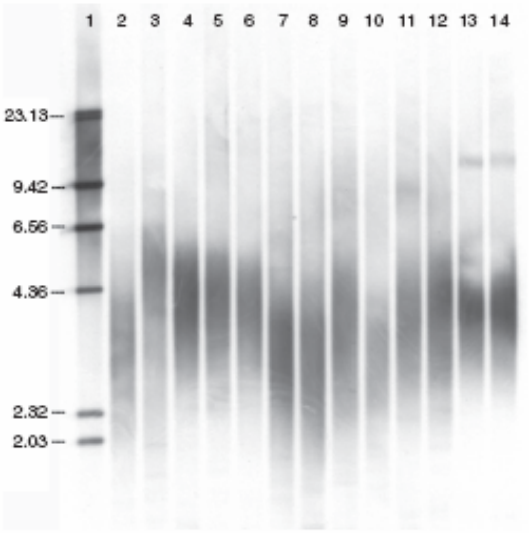

Fig. 2. Southern blot for measurement of telomere length. 1: molecular weight marker, sizes are indicated in kb; 2+3: 12-month-old fish; 4+5: 15-month-old fish; 6+7: 25-month-old fish; 8+9: 27-month-old fish; 10+11: 79-month-old fish.

was detected with chemiluminescence using Anti-Dig-AP Fab Fragment and CDPStar according to the manufacturer's instructions (Roche). Mean telomere length was obtained with telometric1.1.1.jar software (Grant *et al.* 2001) using the formula.

$$\sum(\mathrm{OD})/\sum(\mathrm{OD/MW}),$$

where OD is the optical density and MW is molecular weight.

In order to test the reproducibility of the Telomere Restriction Fragment method, three replicates of each sample were run on different blots, in different lanes. The mean standard deviation (s.d.) of all samples was 123 bp (range = 19–215 bp) or 3.29% (range = 0.45–4.37%), which is comparable to values reported in the literature (0.17–1.4 kb or 2–17%: Counter *et al.* 1992; Gan *et al.* 2001; Grant *et al.* 2001). One sample was run four times on the same blot to determine intragel variation (s.d. = 99 bp or 2.26%). An example of a blot is shown in Fig. 2. Statistical analysis was done using SPSS ver. 16 (SPSS Inc.).

Results

Telomere length

Mean telomere length for individual fish ranged from 2.85 kb to 4.92 kb (Fig. 1*a*). Telomere length did not correlate with age (linear regression, $R^2 = 0.025$), but was significantly different between age groups (ANOVA, $F_{6,17} = 4.24$, $P = 0.009$). *Post hoc* comparisons (Tukey HSD) showed a significant difference between fish of 48 and 27 months of age ($P = 0.04$) and trends towards significant differences between 48 months and 12 months ($P = 0.071$), 25 months ($P = 0.061$) and 79 months ($P = 0.056$).

Discussion

This study tested whether erythrocyte telomere length change can be used as a sublethal ageing tool in fish, as has been proposed for birds (Haussmann and Vleck 2002). A recent study in medaka found a decline of telomere length with age in several tissues (Hatakeyama *et al.* 2008), but these tissues require destructive sampling and thus are not suitable for brood stock management or conservation studies. In contrast, blood sampling is a relatively low-invasive approach from which sufficient DNA can be obtained from small samples because fish erythrocytes are nucleated.

We expected a decline of telomere length with age, as found in bird erythrocytes (Haussmann *et al.* 2003*b*) and most recently several medaka tissues (Hatakeyama *et al.* 2008). Instead, telomere length was very short in all age classes, comparable with the telomere length observed in human cells at the onset of senescence (Baird and Kipling 2004), and did not change with age (Fig. 1*a*). Although our data cover only half the natural life span of sea bass (average life span is 15 years in the wild, but some individuals in the examined brood stock are 24 years old: Fritsch and Fauvel, pers. comm.), because changes in telomere length are generally faster in younger fish (Hatakeyama *et al.* 2008) or birds (Hall *et al.* 2004; Pauliny *et al.* 2006), we should have been able to detect any trend of telomere length change that exists in this species. The lack of telomere length change with age in sea bass erythrocytes requires further investigation, but might be due to high telomerase activity, as detected in medaka and rainbow trout kidney, the main tissue of erythrocyte formation (Klapper *et al.* 1998; Hatakeyama *et al.* 2008).

We did, however, find age-independent differences between the age groups. This was especially evident in older fish, where telomere length was similar within, but different between, age classes (Fig. 1*b*). The breeding regime of our aquaculture population of sea bass makes it likely that most individuals of one age group are siblings and all groups have been kept separately in slightly different rearing conditions (e.g. temperature, feeding regime or stock density: cf. Hatakeyama *et al.* 2008; where rearing conditions were highly controlled). Consequently, two pathways could lead to the age-independent differences between the groups: inheritance and stress response.

Inheritance of telomere length is a multilocus trait that includes loci associated with the expression of telomerase (Baird 2008). In humans, individual-specific telomere length is believed to be established in the zygote and subsequent decrease in somatic cells is attributed to the lack of telomerase activity (Baird 2006). In contrast, telomerase activity is present in most fish tissues throughout the whole life (Klapper *et al.* 1998). Where telomerase activity does occur, the effect of allelic differences in telomerase expression or activity could also have an impact on telomere length that will not be limited to the zygote. In this case, the telomere length of individuals would act as an accumulative record of different telomerase activity and would result in a pattern similar to the one we observed (i.e. more distinct clusters of telomere length for older age-classes).

Stress, including oxidative damage or general stress levels, is known to influence telomere length (Von Zglinicki 2002; Valdes *et al.* 2005; Demissie *et al.* 2006; Matthews *et al.* 2006; Simon *et al.* 2006) and telomerase activity (Epel *et al.* 2004). While the latter study found a decrease of telomerase activity with general (life) stress in human peripheral blood mononuclear cells (PBMC), a positive correlation between telomerase activity and oxidative stress has been found in lens epithelial cells of dog (Colitz *et al.* 2004). Channel catfish PBMC cell lines displayed an upregulation of telomerase activity after few days in culture and a subsequent downregulation after ~4 weeks (Barker *et al.* 2000). Although the response mechanisms are poorly understood, this shows that telomerase activity can be influenced by external factors.

Owing to the varied conditions of rearing for the fish used in this study, individuals of different age groups are likely to experience varying stress factors. Feeding regime and stock density differed between the groups, but seasonal factors might also influence stress levels. For example, the water supply to the fish was taken directly from the sea. Seasonal temperature fluctuations or pollution levels in the water supply might act more strongly on juvenile fish than on adults, exposing fish born at a certain time of the year to more stress than fish born earlier or later.

We believe that a mixture of inheritance and stress response can account for the distribution we observed between the age classes. In this model telomere length in fish is the result of shortening by oxidative damage and replication and lengthening by telomerase activity influenced by individual genetic background and external factors like stress level. Telomere length would then be a record for the life history of each individual. Interestingly, telomere length has been found to correlate with fitness (Pauliny *et al.* 2006) and survival rate (Haussmann *et al.* 2005) in birds. If similar mechanisms are present in fish, telomere length could be a useful tool for brood stock management in aquaculture and wild fishery management.

If the pattern in telomere length that we observed is indeed correlated with experienced stress levels in fish, telomere length could be an important marker in aquaculture, fishery and wildlife management. Different rearing conditions could be tested to find a low stress environment to maximise production of established species. There are also plans to use telomere length as a stress marker in the northern blue fin tuna (*Thunnus thynnus*) to help establish a captive breeding population of this commercially important, but declining species (C. R. Bridges, pers. observ.). In wild populations, telomere length as a stress marker could find wide use in species conservation. For example, for species that inhabit costal waters, such as sea bass, human-induced stress such as pollution or habitat degradation might be detected and counteracted by appropriate measures to secure the survival of decreasing wild populations.

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